



The Role of TSC in Oligodendrocyte Differentiation and Myelination

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The Role of TSC in Oligodendrocyte Differentiation and Myelination

Abstract

Tuberous Sclerosis Complex (TSC) is an autosomal dominant syndrome characterized by epilepsy, intellectual disability, and autism. Recent studies have suggested that white matter abnormalities, including hypomyelination, contribute to the cognitive deficits in TSC patients, but the mechanism has remained elusive.

I used the neuron-specific *Tsc1* knockout mice that display a marked decrease in myelin and show that oligodendrocytes are arrested at immature stages of development *in vivo* resulting in a reduction in the number of myelinating cells. I established an oligodendrocyte culture system and examined the effect of neuron-conditioned media and found that the *Tsc1* mutant phenotype was replicable *in vitro* using medium collected from *Tsc1* knockdown (TSC-KD) neurons, confirming that a secreted signal is responsible for inhibiting differentiation of the oligodendrocytes.

I took an unbiased genome-wide approach and identified Connective Tissue Growth Factor (CTGF) as a putative candidate for the secreted signal. I confirmed that CTGF was upregulated in *Tsc1* mutant neurons and characterized its spatial and developmental expression pattern in our mouse model. *In vitro*, CTGF was sufficient

to inhibit differentiation of oligodendrocytes. The addition of CTGF neutralizing antibody to the TSC-KD neuronal media was able to reverse the suppression of oligodendrocyte maturation, strongly suggesting that CTGF is a major component of the oligodendrocyte inhibitory signal derived from *Tsc* mutant neurons.

Since TSC mutation affects all cells, I investigated the role of TSC in oligodendrocytes. In response to TSC knockdown, oligodendrocytes demonstrate an upregulation of cellular stress marker. I also found a decrease in myelin protein genes, a finding that offers interesting implications for the role of TSC in hypomyelination. Furthermore, I expanded my research into Zellweger disease, a syndrome that involves TSC in its neuropathological manifestations including white matter deficits, and found that localization of TSC to the peroxisome is a critical factor in neuron development.

Together, this body of work developed new approaches in Tuberous Sclerosis research in the brain to investigate a previously under-appreciated aspect of TSC pathology – myelination. I have demonstrated that the TSC pathway has important roles in neuron-oligodendrocyte communication and emphasize the critical importance of neuron-derived signals in the establishment of myelination.

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Chapter 1

Introduction

Tuberous Sclerosis Complex

Tuberous Sclerosis Complex (TSC) is a multisystem, autosomal dominant syndrome in which 90-95% of affected individuals have central nervous system (CNS) involvement. Neurologically, the syndrome can manifest with intellectual disability, behavioral abnormalities, autism spectrum disorders (ASD), and seizures (Curatolo et al., 2008). Epilepsy occurs in 80% to 90% of all patients, often with medically refractory seizures. Close to 45% of patients have mild-to-profound intellectual disabilities while ASD occurs in up to 50% of patients (Curatolo et al., 2008; Jeste et al., 2008). TSC can be diagnosed in the pre- or perinatal period (Datta et al., 2008), and many neuropathological features such as cortical tubers are present by the second trimester *in utero* indicating that neurological manifestations of the disease develop during the embryonic period (Raju et al., 2007; Levine et al., 2000). Clinical signs can be markedly variable even within individuals harboring same mutation, some presenting with minimal symptoms while others are severely affected even within the same family.

The neuropathological findings in the brain usually take the form of (1) subependymal nodules, (2) subependymal giant cell astrocytomas (SEGA) and (3) cortical tubers (DiMario et al., 2004). Subependymal nodules are lesions found along the wall of the lateral ventricles in the brain. In 5-10% of cases, these “benign” lesions can grow into SEGAs that block the circulation of cerebrospinal fluid resulting in hydrocephalus. Tubers are made up of a collection of abnormally large neurons and glia, and are most commonly found in the cerebral cortex. It has been proposed that the presence of cortical tubers contribute to the severity of the disorder, but studies have presented conflicting findings on which aspects of the tubers are most critical indicators. More recent studies indicate that the tuber volume is a better reflection of the severity of cognitive impairment than tuber number alone, and also that the location of the tubers (frontal/occipital/temporal/cerebellar) has differential associations with comorbid neuropsychiatric disorders (Eluvathingal et al., 2006; Bolton et al., 2002; Jansen et al., 2008). However, it has become increasingly clear that characterizing the tubers alone cannot explain the variable clinical symptoms, and careful investigation into the molecular mechanisms of TSC is necessary.

TSC1/2 Protein Complex

TSC affects approximately 1 in 6000 newborns and is caused by a mutation in the *TSC1* or *TSC2* genes that encode for hamartin and tuberin, respectively. *TSC1* (on chromosome 9) and *TSC2* (on chromosome 16) are tumor suppressor genes that integrate extrinsic and intrinsic signals of the cellular energy status and growth.

These two proteins bind each other to form a GTPase activating protein (GAP) complex that plays a critical role in regulating protein synthesis and controlling cell size in response to growth factor stimulation (Kwiatkowski and Manning, 2005). Both proteins are required for the proper function of the complex, and thus a mutation in either gene is sufficient to cause the clinical disease. TSC1 is required to stabilize TSC2 and prevent its degradation. On the other hand, the functional GAP domain resides in TSC2, making each protein obligatory for each other's functional role (Review in Kwiatkowski and Manning, 2005). In fact, studies in *Drosophila* have shown that the *Tsc1;Tsc2* double mutants phenocopy either single mutants and that overexpression of both proteins is required to render a gain of function phenotype (Tapon et al., 2001; Gao et al., 2001). Nonetheless, patients with *TSC2* mutations have a worse overall prognosis than those with *TSC1* mutations (Dabora et al., 2001), and the conditional *Tsc2* knockout mouse model has a more severe phenotype than the conditional *Tsc1* knockout in the same conditional genetic background (Zeng et al., 2010). These differences could be due to the two proteins having additional independent functions. Another possibility is that, although both TSC1 and TSC2 are subject to ubiquitin-mediated degradation if not bound to each other, some enzymatic activity of TSC2 remains and is able to carry out some of its function before its degradation. TSC1, which has no such catalytic domain, would be ineffective in suppressing any mTOR activity on its own (Benvenuto et al., 2000)

Investigations into the protein interactors of TSC1/2 have begun in non-neuronal cell lines (Nellist et al., 2005; Rosner et al., 2008; Guo et al., 2010), but the question of which proteins interact with the TSC1/2 complex in CNS cells at

different times during development have yet to be explored. One function of the TSC1/2 complex is to inhibit the Ras family GTPase, Rheb, the downstream targets of which include the serine-threonine kinase mammalian target of rapamycin (mTOR), a master regulator of protein synthesis (Figure 1.1). mTOR kinase exists in two distinct functional complexes, mTOR Complex 1 and Complex 2, defined by two groups of binding partners (Huang et al., 2009). mTORC1 is bound strongly and is quickly inhibited by rapamycin while mTORC2 inhibition requires prolonged rapamycin treatment, which blocks mTORC2 assembly (Sarbasov et al., 2006). Without the functional TSC complex, mTORC1 is hyperactive, resulting in constitutively phosphorylated ribosomal S6 protein, disinhibited protein synthesis, and subsequent cell growth (Sarbasov et al., 2006; Wullschleger et al., 2006).

Upstream Regulation of TSC

The TSC1/2 complex can be regulated post-translationally by several major signaling pathways in cells: PI3K-Akt, ERK and AMPK. The best-characterized function of the TSC1/2 complex is as a downstream target of the phosphatidylinositol 3-kinase (PI3K) pathway that becomes activated upon the binding of growth factors (e.g. IGF or BDNF). Activated PI3K leads to recruitment of PDK1 and the serine/threonine protein kinase Akt, and subsequent phosphorylation/activation of Akt by PDK1. Activated Akt negatively regulates TSC by directly phosphorylating TSC2 on five consensus sites on human TSC2 (Potter et al., 2002; Dan et al., 2002; Manning et al., 2002; Inoki et al., 2002). A second kinase that can phosphorylate and inhibit TSC2 is the extracellular signaling-regulated

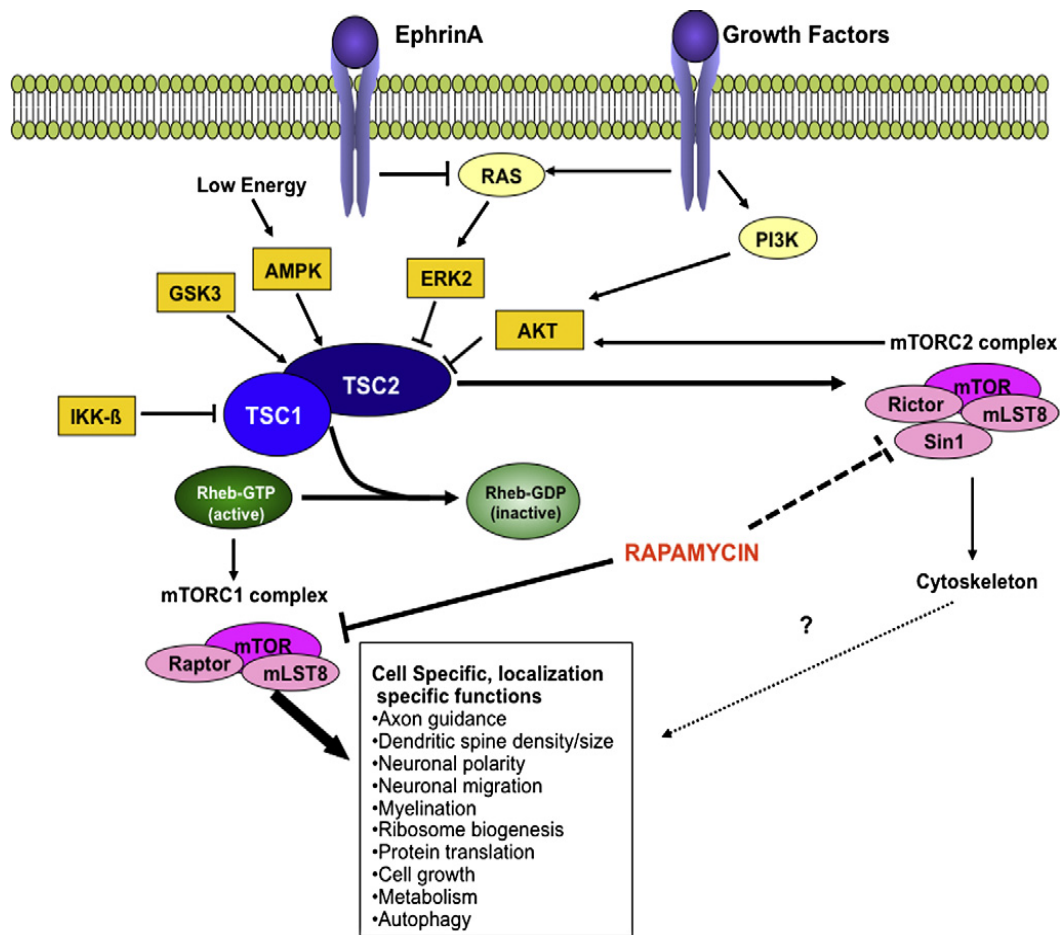


Figure 1.1. TSC mediated signaling in the CNS. This cartoon of TSC mediated signaling has been simplified to highlight the demonstrated biologic roles for TSC mediated mTOR signaling in the nervous system (Han et al., 2011).

kinase (ERK) (Ma et al., 2007). ERK phosphorylation of TSC2 appears to be particularly important for EphA-receptor mediated regulation of the TSC1/TSC2 protein complex (Nie et al., 2010). Both active Akt and ERK levels are found to be high in TSC-related cortical tubers and SEGAs, and the inhibition of TSC2 by these kinases has been proposed to represent a posttranslational mechanism that may further amplify the loss of the first allele of the TSC gene (Ma et al., 2007; Han et al., 2004). In addition, AMP-activated protein kinase (AMPK) can phosphorylate TSC2 on a different set of residues than Akt and ERK and potentially increase the ability of TSC1/2 to inhibit the mTORC1 activity, thereby protecting cells from excessive energy use during low energy states (Inoki et al., 2003; Hahn-Windgassen et al., 2005). TSC1 is also negatively regulated by IKK-beta, which physically interacts with and phosphorylates TSC1 at Ser487 and Ser511 in response to inflammatory pathway activation (Lee et al., 2007). Because TSC1:TSC2 functions as a heterodimer, regulation of either protein most likely affects its overall activity level. However, relatively little is known about the post-translational modifications affecting the TSC1 protein and the hierarchy of the regulatory modification on the TSC1/2 complex, particularly those involving AMPK and IKK. Furthermore, none of these posttranslational modifications appear to affect the GAP activity of TSC2 per se, although they unlikely affect the ability of the TSC1/2 complex to act as a Rheb-GAP in cells.

Downstream of TSC: mTORC1 and 2

When active, TSC2 inhibits Ras family GTPase Rheb by stimulating the conversion of Rheb-GTP to Rheb-GDP. Downstream targets of Rheb include the serine-threonine kinase mammalian target of rapamycin (mTOR), a central regulator of protein synthesis. mTOR kinase exists in two distinct functional complexes, mTOR Complex 1 and mTOR Complex 2, defined by two groups of binding partners. mTORC1 is comprised of the core essential components Raptor and LST8, while mTORC2 contains Rictor, LST8, and SIN1. mTORC1 is bound strongly and is quickly inhibited by rapamycin, while mTORC2 inhibition requires prolonged rapamycin treatment, which blocks mTORC2 assembly (Sarbasov et al., 2006). However, rapamycin does not fully inhibit mTORC1 function, with some downstream targets being more sensitive than others (Choo et al., 2008). mTORC1 phosphorylates and activates ribosomal S6 kinases (S6K1 and S6K2) and inhibits the translational regulator 4E-BP1 – both events that positively regulate translation of 5' capped mRNAs. mTORC1 phosphorylates S6K1 on Thr389, resulting in phosphorylation of its downstream effectors that increase mRNA translation (Ruvinsky et al., 2005). Activation of S6Ks leads to phosphorylation of ribosomal protein S6, elongation factor 2 kinase (eEF-2K), programmed cell death protein 4 (PDCD4), and eIF4B, all of which result in increased protein synthesis (Ma et al., 2009; Raught et al., 2004; Wang et al., 2001; Dorrello et al., 2006).

Unphosphorylated 4E-BP1 is bound to eukaryotic initiation factor 4E (eIF4E), inhibiting its association with the eIF-4F cap-binding complex, thereby blocking translation initiation (Pause et al., 1994). When phosphorylated by mTORC1, 4E-BP1 dissociates from the eIF4E complex, initiating mRNA translation (Dowling et al.,

2010; Gingras et al., 2001). Thus, without the functional TSC complex, mTORC1 is hyperactive, resulting in constitutively phosphorylated S6 protein, disinhibited protein synthesis, and subsequent cell growth (Wullschleger et al., 2006; Ruvinsky et al., 2006).

As a central regulator of cell growth, mTORC1 is sensitive to nutrient and redox states of the cells, and more recently has been shown to be specifically responsive to amino acids through a not yet well defined pathway involving the Rag GTPases (Kim et al., 2008; Sancak et al., 2010; Sancak et al., 2008). The presence of amino acids alters the nucleotide-bound state of a heterodimeric Rag complex at lysosomal membranes, and this creates a docking site for mTORC1 (Sancak et al., 2010). Once at the lysosomal membrane, mTORC1 encounters Rheb, although it is undetermined how or if Rheb is targeted to the same endomembranes as mTORC1 and Rag proteins. Whether neuronal mTORC1 has specific function at the lysosome has yet to be investigated, and it would be interesting to elucidate other cellular localization sites of TSC1/2 and mTORC1 and their relevance to the function of this signaling pathway. I will begin to explore the importance of TSC1/2 localization in neuronal development in Chapter 5.

Our understanding of mTORC2 is nascent when compared to mTORC1 (especially in the CNS), but it is emerging as a critical component of the PI3K/mTOR pathway. While TSC1/2 negatively regulates mTORC1, it promotes mTORC2 activity in a Rheb-independent manner that might involve the direct binding of the TSC1/2 complex to components of the mTORC2 complex (Huang et al., 2009; Huang et al., 2008; Yang et al., 2006). Once active, mTORC2 phosphorylates and activates AKT,

leading to phosphorylation of its downstream effectors including TSC2 (Hietakangas et al., 2008). There also appears to be crosstalk between mTORC1 and mTORC2, as S6K1 phosphorylates and inhibits Rictor (Julien et al., 2010). Interestingly, loss of TSC1 or TSC2 leads to a unique cellular scenario in which mTORC1 is activated and mTORC2 is attenuated. An important implication of these findings is that the ideal treatment for loss of TSC1/2 may require not only mTORC1 inhibition but also mTORC2 activation. As most of these initial studies were performed in non-neuronal cells, it has yet to be seen whether the pathway is conserved in the CNS and how the intricate balance between the two mTOR complexes affects neuronal function. Studies in the last few years have begun to shed light on the role of these proteins in several aspects of neural development and function, and it is becoming clear that TSC1/2 protein complex is a master regulator of neuronal connectivity, essential in synapse, plasticity, and axon guidance (Reviewed in Tsai and Sahin 2011). In my thesis, I will address another very important aspect of connectivity: myelination.

Hypomyelination in TSC patients and mice

Much of TSC/mTOR pathway research in the central nervous system has been focused on neurons and astroglia, while roles in oligodendrocytes development and myelination have not yet been fully illuminated. Until recently, the literature has focused on the interplay between epilepsy and cortical tubers as the source of the neurodevelopmental disabilities in TSC. As a result, neuronal abnormalities have long been considered the basis of TSC neuropathology with regard to seizures, behavioral disorders, and autism. Tubers are made up of a

collection of abnormally large neurons and glia and are most commonly found in the cerebral cortex and it has been proposed that the presence of cortical tubers contribute to the severity of the disorder. However, studies have presented conflicting findings on which aspects of the tubers are the most critical indicators. More recent studies indicate that the tuber volume is a better reflection of the severity of cognitive impairment than tuber number alone, and also that the location of the tubers (frontal/occipital/temporal/cerebellar) has differential associations with comorbid neuropsychiatric disorders (Eluvanthinal et al., 2006; Bolton et al., 2002; Jansen et al., 2008). In addition, a role for astrocytes also has been demonstrated (Uhlmann et al., 2002a and b) while white matter abnormalities have received surprisingly little attention. However, growing evidence, from abnormal white matter on neuroimaging of TSC patients to deficits in axonal integrity and myelination in mouse models, supports the hypothesis that TSC1/2 proteins play crucial roles in white matter development and subsequent TSC manifestation in the CNS (reviewed in Tsai et al., 2011; Han et al., 2011).

The evidence of whether tubers directly contribute to CNS abnormalities is conflicted, but what has become clear is that white matter, myelin, abnormalities coincide best with autism spectrum disorders and cognitive function. Myelination defects are commonly observed in the TSC brain, both focally within tubers and more diffusely (Makki et al., 2007; Ridler et al., 2001; Zikou et al., 2005). Focal white matter deficits are frequently in the subcortical region underlying tubers, highlighted by ectopic neurons, loss of axons, giant cells, and large astrocytes. More diffusely, this congenital defect persists throughout adulthood, infiltrating

numerous major intrahemispheric tracts bilaterally causing approximately 15% reduction in white matter volume (Ridler et al., 2007). Furthermore, recent diffusion tensor imaging studies indicate that TSC patients have occult damage in the normal appearing white matter and that this damage may contribute to neurocognitive disability in these patients (Makki et al., 2007; Piao et al., 2008; Yu et al., 2009; Krishnan et al., 2010; Peters et al., 2012). Despite its significance, it has been unclear whether the defect in myelination is a cell-autonomous affect of oligodendritic TSC/mTOR or due to Tsc-deficient neuronal dysfunction. The significance of white matter abnormality in neurodevelopmental disorders prompt thorough investigation into the origins of deficits. We have recently gained important clues by creating transgenic disease model mice.

Mouse Models of TSC

Sophisticated analysis of the neuronal function of TSC1/2 genes *in vitro* and in animal models has revolutionized our understanding of the disease mechanisms and potential treatment options. Most neuropathological features of TSC develop embryologically, making early intervention and treatment even more critical. There are so far 393 *TSC1* and 1118 *TSC2* known unique allelic variants involving almost all of the exons of *TSC1* and *TSC2*, arising from nonsense, missense, insertion, and deletion mutations. In addition to these complexities, the multitudes of variance in severity of the diseases and abundant number of effected downstream pathways make genotype-phenotype correlations difficult. One major endeavor to overcome these difficulties has been to generate a proper animal model, and although one has

yet to completely mimic the spectrum of phenotypes seen in humans, (e.g. cortical tubers, subependymal nodules or SEGAs), conditional knockouts of the *Tsc1* or *Tsc2* genes have established firm molecular basis of the disease.

The first models of TSC established that haploinsufficiency of either Tsc genes causes neurocognitive deficits such as impaired hippocampal-dependent learning, social behavior, synaptic plasticity, learning and memory (Ehninger et al., 2008; Goorden et al., 2007). *Tsc2* heterozygous mice also exhibit abnormal mother-pup interaction as measured by ultrasonic vocalizations (USV), establishing these mice as potential models of autism (Young et al., 2010). These neuropsychiatric abnormalities are present without obvious concomitant neuropathological alterations, prompting more rigorous investigation of subtle molecular and circuitry level changes as well as the need for better models.

One important observation made of the *Tsc1*^{+/-} and *Tsc2*^{+/-} mice was the increase in the numbers of astrocytes (Uhlmann et al., 2002a). As homozygous loss of *TSC1* or *TSC2* results in prenatal death, Uhlmann and colleagues generated *Tsc1*^{flox/flox};*GFAP-Cre* mice to specifically inactivate Tsc1 in astrocytes to study the impact of complete loss of TSC in these cell types (Kobayashi et al., 1999; Uhlmann et al., 2002b). *Tsc1*^{flox/flox};*GFAP-Cre* mice demonstrated up to six-fold increase in GFAP-immunoreactive cells and subsequent enlargement of some cortical regions such as the hippocampus accompanied by alterations in neuronal organizations (Uhlmann et al., 2002b). Additionally these mice developed electroencephalographically confirmed seizures at two months of age but they failed to mimic additional symptoms of TSC such as cortical tubers or cortical lamination

defects. More recently, Zeng et al. found that rapamycin treatment blocked mTOR activity, prevented progressive astrogliosis, abnormal neuronal organization, development of epilepsy and premature death in these mice (Zeng et al., 2008).

Deletion of *Tsc2* from radial glial precursors cells using *hGFAP-Cre* transgenic mice results in lamination defects, cortical enlargement, astrogliosis as well as myelination defects [88]. *Tsc2^{flox/flox};hGFAP-Cre* mice exhibited severe compromise in survival and profound seizure episodes, suggesting again that cortical tubers are not necessary for the observed phenotypes but other neuropathological features such as hypomyelination may be a critical contributor to the prognosis of the disease.

Myelination defects were also seen in neuron-specific knockout of *Tsc1*. These mice were generated using *Cre* recombinase under the *Synapsin-1* promoter (*SynI-Cre*), effectively deleting *Tsc1* in postmitotic neurons (Meikle et al., 2007). *Tsc1^{flox/flox};SynICre* mice are viable perinatally, but develop tremor and hyperactivity beginning the second week of life and die starting approximately four weeks postnatally. These mice exhibit several neuropathological abnormalities similar to those seen in TSC patients including enlarged dysplastic neurons throughout the cortex, hippocampus, and other subcortical grey matter regions as well as spontaneous seizure episodes. mTORC1 inhibitor treatment starting P7 has reversed some neuroanatomical abnormalities associated with clinical TSC including reduction in neuron size and improvements in biochemical/signaling profiles, as well as clinical improvements in body weight, clasping behavior, tremor, seizures and kyphosis (Meikle et al., 2008). Furthermore, when animals are taken off treatment most of the clinical improvements remained intact for at least two

more weeks, indicating that regulation of neuronal mTORC1 is critical not only during neurodevelopment but also for long term maintenance of neuronal function. In addition, the hypomyelination phenotype was prevented with early treatment of rapamycin, highlighting the importance of neuronal mTOR in oligodendrocyte development and/or myelination.

Each of these cell-specific mouse models allowed the researchers to investigate the intrinsic functions of TSC in the respective cell types. Nonetheless perhaps one of the most intriguing phenotype has been the hypomyelination seen in the neuron-specific knock out mouse in which the oligodendrocytes are genetically normal, allowing us to begin exploring the importance of TSC in neuron-oligodendrocyte interactions, a novel approach in the field of TSC. In this thesis, I will focus on the respective roles of *TSC* in both neurons and oligodendrocytes.

TSC in Oligodendrocyte development

Previous studies show that proliferation, differentiation and myelination of oligodendrocyte precursor cells (OPCs) are independently controlled, and that the latter sequences of events are not default stages of its prior. Investigations of mechanisms underlying these processes established that proliferation and differentiation are clearly separate events regulated by distinct factors. Of these, a widely accept factor is neuron-synthesized insulin-like growth factor-1 (IGF-1) that has been shown to affect all three aspects of oligodendrocyte development (Stangel et al., 2002; D'Ercole et al., 2002). Interaction of IGF-1 with IGF-1R on oligodendrocytes result in rapid transcription and *de novo* protein synthesis in the

oligodendrocytes through PI3K/Akt, mTOR, and MEK/ERK pathway activation as shown by respective pharmacological inhibition of each pathway component (Bibollet-Bahena et al., 2009). OPCs' proliferative potential appears to be dependent on extrinsic cues such as platelet-derived growth factor (PDGF), thyroid hormone (TH) and retinoic acid (RA), but their intrinsic modulations such as cell cycle inhibitors p27^{kip1} and p21^{cip1} are also necessary to regulate the number of divisions and to initiate differentiation, respectively. Both axons and astrocytes are sources of these factors that are critical in survival, proliferation and differentiation of the oligodendrocytes. For instance, PDGF, secreted by neurons and astrocytes promotes proliferation and survival of the OPCs (Richardson et al., 1988; Yeh et al., 1991; Barres et al., 1992). OPCs will proliferate indefinitely with addition of PDGF in culture, but when growth factors such as TH are added, the OPCs will divide a fixed number of cycles prior to differentiating, showing that extrinsic and intrinsic factors are carefully coordinated *in vivo* (Barres et al., 1994; Durand et al., 1998; Gao et al., 1998). Interestingly, *TSC1* overexpression has been associated with reduced cell growth and increased levels of p27^{kip1} while loss of p27^{kip1} contributes to increased cell proliferation and tumorigenesis in Rat1 cells (Miloloza et al., 2000; Sherr et al., 1995; Missero et al., 1996). OPC proliferation also appears to be density-dependent via unknown pathways (Zhang and Miller 1996). It remains yet to be seen whether loss of functional TSC would alter proliferative rate of oligodendrocytes by modulation of aforementioned pathways.

Differentiation of OPCs is further regulated by the Notch signaling pathway, whose ligands Jagged1 and Delta1 are able to inhibit differentiation of OPCs without

impairing their proliferative potential (Wang et al., 1998). More recent evidence has shown that inhibiting γ -secretase, a protease for Notch-1 activation, greatly improved differentiation and subsequent myelination of the oligodendrocytes in culture (Watkins et al., 2008). Generation of mature myelinating oligodendrocyte requires another axon-derived signaling pathway, Neuregulin-1/ErbB, which appear to influence morphological changes in oligodendrocytes and have long been implicated in many other important aspects of myelinating cell generation (Vartanian et al., 1994). However, neuregulin has recently been found to have differential role in the peripheral nervous system versus the central nervous system, further emphasizing the need to study oligodendrocyte specific processes (Brinkmann et al., 2008; Figure 1.2 for Review of Oligodendrocyte morphology).

In addition to these complexities is the number of candidates that may ultimately modulate the myelination process. These candidates include Nerve Growth Factor (NGF), which has been shown to inhibit myelination of TrkA-expressing neurons, and polysialic acid-neural cell adhesion molecule (PSA-NCAM), whose inhibition increases the number of myelinated axons *in vivo* (Chan et al., 2004; Charles et al., 2000). Similar to diverse expression of these molecules in the brain, myelination initiation also seems to be a region-specific process. For instance, the onset of myelination in the fiber tracts of the rat spinal cord varies from P2 to P14, in rostral-caudal gradient (Schwab and Schnell 1989). In the optic nerve, the oligodendrocytes express MBP and PLP in chiasm-to-eye gradient, while the actual myelination occurs in reverse gradient (Colello et al., 1995). Molecularly, transcription of major myelin-specific genes such as *Mbp*, *Plp1* and *Mag* are

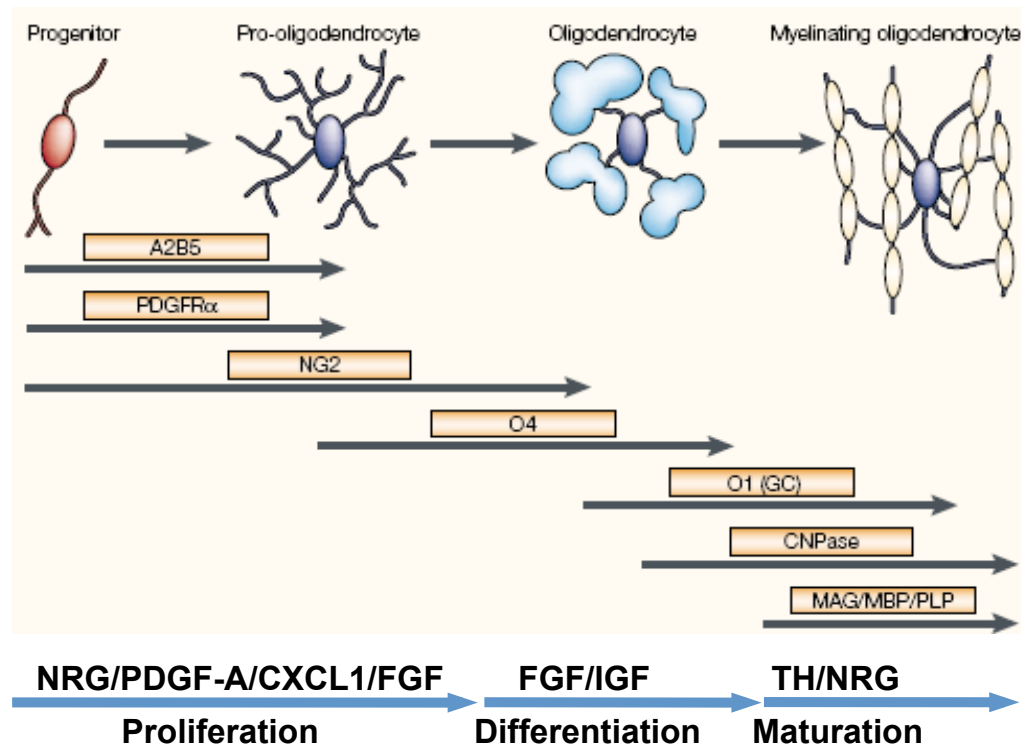


Figure 1.2. Morphological and antigenic markers for oligodendroglia during development. The differentiation of oligodendrocytes from their progenitors follows a stepwise morphological transformation from bipolar progenitors to pro-oligodendrocytes bearing multiple processes (immature), membrane-sheath-bearing mature oligodendrocytes, and, finally, myelinating oligodendrocytes. Accompanying this morphological change is the sequential expression of molecular markers: A2B5 antigen, PDGFR and chondroitin sulphate proteoglycan NG2 in progenitors, O4 antigen in pro-oligodendrocytes, galactocerebroside (GC or O1 antigen) and CNPase in mature oligodendrocytes, and most myelin proteins — MAG, MBP, and PLP — in myelinating oligodendrocytes. These morphological and expression changes may be in response to extrinsic factors (modified from Zhang 2002)

regulated by the basic helix-loop-helix transcription factor, Olig1 in the brain (Xin et al., 2005). OPCs of *Olig1*-null mice proliferate, differentiate, and migrate properly but fail to initiate myelination despite possessing branched processes and having contact with the axons, highlighting the presence of distinct pathway for myelinogenesis (Xin et al., 2005). Individually, oligodendrocytes constitute morphologically, functionally, and biochemically heterogeneous populations of cells depending on the type of axon being myelinated. Oligodendrocyte type I and II express higher levels of carbonic anhydrase 11 and myelinate mainly small diameter fibers, while type III and IV express L and S isoforms of myelin associated glycoprotein (MAG) and myelinate large diameter fibers (Butt et al., 1995 and 1998).

Transcription factors such as Olig1 or Olig2 are usually regulated by neuron- or astrocyte-secreted signals, but oligodendrocytes also can secrete factors to “prepare” the axons for myelination such as clustering of the sodium channels, pointing to the importance of proper biochemical signaling pathways within the OPCs (Kaplan et al., 1997). However, despite the active role of the oligodendrocytes in neuronal integrity, the major intracellular signaling pathways are relatively understudied and oligodendrocytes have been viewed as passive recipients of axon derived signals. Oligodendrocytes can increase axonal stability, induce local accumulation of neurofilaments within the axons, and secrete factors that increase the survival of neurons *in vitro* via the PI3K/Akt signaling pathway (Sanchez et al., 2000; Brady et al., 1999; Wilkins et al., 2003).

These complexities in oligodendrocytes development and myelination necessitate a thorough and careful investigation into the cause of hypomyelination in TSC disease, and the reciprocal interactive relationship between neurons and oligodendrocytes. The previous study characterizing reduced MBP staining did not address which aspect of oligodendrocyte development may be altered in TSC-deficient animals (Meikle et al., 2007). Given the multitude of stages and levels at which the development and myelinating properties of the oligodendrocytes can err, the first step of the investigation in identifying the most probable pathway is to define the timing and the source of signaling deficiency.

Possible role of TSC in oligodendrocytes

One possible cause of hypomyelination in TSC patients is cell death that could be intrinsically activated by the TSC/mTOR pathway. Our laboratory has previously shown that Tsc loss results in mTOR dependent endoplasmic reticulum (ER) stress response at baseline, and upon treatment with stress-inducing agents such as Thapsigargin, the Tsc2-deficient cells exhibited lowered threshold for induction of unfolded protein response (UPR) regulated genes and mitochondrial cell death pathways (Di Nardo et al., 2009). More importantly, the lack of Tsc activity led to increased expression of pro-apoptotic transcription factor CHOP (C/EBP homologous protein), production of reactive oxygen species (ROS), and susceptibility to apoptosis. This result was replicated in numerous cell types including neurons, HEK293T, and mouse embryonic fibroblasts. Given the reproducibility across the cell types, TSC deficiency may have similar effects on

oligodendrocytes, leading to cellular stress and vulnerability to apoptosis. Although this may not be the source of hypomyelination in the neuron-specific knockout mouse, it is an important aspect of oligodendrocyte development to investigate since TSC patients have TSC-deficiency in all cells. Considering the lower threshold for cellular vulnerability to apoptosis in OPCs, *Tsc1*-deficiency in the OPCs may have an additive effect resulting in much higher likelihood for cell death. As the total number of mature oligodendrocytes is determined by a combination of OPC proliferation, survival, and differentiation, and it is possible that deficient TSC/mTOR function in OPCs decreases the progenitor pool available to mature into myelinating oligodendrocytes.

Indeed, related proteins of the TSC/mTOR pathway have recently emerged as critical regulators of OPC development, myelination and maintenance. For instance, over-expression of constitutively active Akt in OLs (*Plp*-Akt-DD mice) results in hypermyelination, which can be blocked by rapamycin treatment (Flores et al., 2008; Narayanan et al., 2009). However, it is important to remember that Akt has many other substrates in the cell, and that TSC plays a critical role in established pathways such as growth factor phosphatidylinositol 3-kinase (PI3K)-Akt with widespread downstream targets in all cell types. Therefore, it is most likely that it also alters intrinsic function of oligodendrocytes. In the same study, rapamycin treatment of wildtype mice for 3 weeks starting at P21 also reduced myelination, indicating mTOR activity is necessary for myelination during this period of development (Narayanan et al., 2009). Another study using conditional deletion of the gene *Phosphatase and tensin homolog (Pten)* in oligodendrocytes (either

Pten^{f/f}Olig2-Cre; or *Pten^{f/f}Cnp1-Cre*) results in hypermyelination despite the lack of relative increase in OL numbers (Harrington et al., 2010; Goebbels et al., 2010). Moreover, *Pten^{f/f}Olig2-Cre* mice exhibited progressive demyelination and axonal degeneration by yet unidentified mechanism, highlighting the importance of this pathway in maintenance of myelin beyond development.

While all of these studies focused on molecules upstream of TSC1/2, other studies have focused on molecules downstream of Tsc1/2. Deletion of *Rheb1* in both neurons and OLs (using the Nestin-Cre deleter line) led to hypomyelination (Zou et al., 2011). In contrast, overexpression of a constitutively active Rheb transgene in both neurons and OLs increased expression of myelin proteins early on (P7), although no difference was observed by P14. Using Nestin-Cre as the deleter line makes it impossible to determine whether the effect on myelination is due to the expression of Rheb1 in oligodendrocytes or neurons, a disadvantage the authors concede. For example, the early increase in myelin proteins may be due to Rheb activity in oligodendrocytes, while the later normalization of myelination may be due to the Rheb activity in neurons. These discrepancies in the studies that investigate the same pathway highlight the importance of illuminating the oligodendrocyte-specific loss of *Tsc1*, given its most direct correlations to the human disease. Therefore, detailed understanding of the role of TSC/mTOR pathway in CNS myelination by studying both the OL-specific loss of Tsc function and neuronal-specific loss of *Tsc1* function is necessary to delineate cell-specific and development specific roles for the TSC1/2 protein complex in the development of white matter.

Much of the focus in the field has been on astrocytes and neurons, perhaps because of their direct contributions to epilepsy and tuber formation. Nonetheless, the correlation between the severity of cognitive impairments and degree of hypomyelination has brought to surface that the TSC/mTOR pathway is also critical in development and function of oligodendrocytes. Therefore I chose to study the cell-autonomous and indirect effects of the loss of the pathway on these cell types to illuminate the pathogenetic mechanisms underlying hypomyelination, and ultimately, neurocognitive deficits. The goal of my thesis is to understand the molecular mechanisms underlying hypomyelination in TSC. More specifically, I have investigated the respective contributions of neuronal TSC and oligodendrocyte TSC, and how each of their roles is critical in normal differentiation and myelination of oligodendrocytes.

In order to achieve this, I employed the following strategy:

1. I first thoroughly characterized the myelination deficit phenotype in our mutant mice model.
2. I established an *in vitro* culture system to confirm that a neuronal secreted factor is sufficient to result in the arrest in differentiation.
3. In collaboration with Drs. Daniels Geschwind and Dennis Wall, we took an unbiased approach to identify the responsible neuronal molecule.
4. I showed that this molecule is necessary and sufficient to result in the arrest in differentiation seen *in vitro*.
5. I explored some of the consequences of TSC loss in oligodendrocytes *in vitro*.

6. I show that localization of TSC is also important for its function in the neurons.

This body of work is the first known study to investigate the neuron-glial interaction in TSC, and provides evidence that TSC/mTOR-regulated paracrine signaling cells is an important aspect of neurodevelopment. The goal of this work was to illuminate the aspects of TSC pathway that has not yet been investigated and by doing so, it is the hope that we are one step closer to developing better targeted therapeutic treatments for the numerous debilitating hypomyelinating diseases.

Chapter 2

Materials and Methods

Mouse

All experimental procedures were performed in compliance with animal protocols approved by the IACUC at Children's Hospital, Boston.

All mice were mixed-strains, derived from C57BL/6, CBA, 129S4/SvJae strains. I used the following mice strains:

-- *Tsc1^{flox/flox};SynI-Cre* (*Tsc1* KO in neurons)

SynI-Cre mice allow us to knockout a gene of interest (*Tsc1*) only in post-mitotic neurons (Zhu et al., 2001). Homozygous knockout of many of the strains we use are embryonically lethal, so to generate mice missing *Tsc1* in neurons, we crossed the *Tsc1^{f/f}* mice to the *SynI-Cre* mice, allowing us to generate conditional, brain-specific *Tsc1* knockout mice. *Tsc1^{flox}* allele has loxP sequences flanking exons 17 and 18, resulting in deletion the allele in *SynI-Cre* positive neurons upon recombination. Deletion of the two exons has been shown to generate no *Tsc1* protein expression in the (Kwiatkowski et al., 2002). These mice were maintained according to the *Tsc1^{f/f} SynI-Cre⁺* males and *Tsc1^{f/f}* female breeding scheme.

--*PLP-cre(ER)*

These mice have a tamoxifen inducible Cre-mediated recombination system driven by the mouse Plp1, proteolipid protein (myelin) 1 promoter. By crossing these mice with *Tsc1^{flox/flox}* mice, I can ablate Tsc1 in oligodendrocytes and Schwann cells only. Tamoxifen treatment (1mg/40g) will also induce Cre recombination in developing embryos of the treated mothers and in cultured cells from the transgenic mice, generating spatially and temporally controlled expression of our gene product during development.

--*Tsc1^{flox/flox}Plp-Cre⁺(ER)*

We have generated these mice using the two aforementioned lines of mice. To target the oligodendrocytes only, I will tamoxifen treat the mothers when they are nursing the newborn pups at P1. This treatment has been found effective previously. Although there is no data presented from these mice, they are being generated to follow up with our study.

--*PLP-eGFP*

Mice expressing enhanced green fluorescent protein (eGFP) under the control of proteolipid protein (*plp*) gene promoter provide a robust mouse model for the study of oligodendrocytes since PLP is a major component of myelin and it is produced mainly by the oligodendrocyte cell lineage.

We did not discriminate the sex of the animals for the studies. All animals were kept in cages with no more than five adults total, or two adults with a nursing litter. The nursing litter was weaned from lactating females at P21. All animals were provided with food and water *ad libitum*.

Rapamycin treatment

We injected 6 mg/kg intraperitoneally every other day beginning at P7 until sacrificed. This treatment timing and dosing has been shown to be effective in reversing the hypomyelination phenotype previously (Meikle et al., 2008).

Perfusion

For histological studies of the brain, I perfused the mice using the following methods for all ages. I first injected Avertin (125-240 mg/kg) into the left or right abdominal muscle using a 1cc syringe. After verifying that the drugged mouse is unresponsive by pinching the tip of its tail I cut a V-shape extending up on either side of the sternum (bottom plate of the rib cage) to uncover the skin and fur. I extended this V-cut through the tissue layer and rib cage and flipped the resulting section up and pin it down nearby the mouse's head to reveal the liver (red) and above, the beating heart. I then snipped the right atrium and observed the blood flowing out, quickly penetrating the left ventricle with the butterfly-winged adapter needle, making sure that the needle is sufficiently lodged inside the ventricle, and lay the adapter cord across the mouse's body. Using the attached syringe, I slowly pumped 15-20mL of PBS into the left ventricle to flush out the mouse's tissues. Once the liver turns white/cream in color and the fluid exiting the right atrium runs clear, I removed the PBS syringe from the adapter and replace it with PFA, slowly pumping 15-20mL of PFA into the left ventricle to fix the mouse's tissues. Once the mouse is properly perfused, I dissected out the brain, the optic nerve, and the sciatic nerve to store the tissue in 4% PFA over night on the rocker in the cold room. The

following day, I rinsed the tissue with PBS three times, 30 minutes each. After the last wash, I cryoprotected the tissue by immersing the tissue in 30% sucrose overnight at 4°C, or until the brain sunk. The cryoprotected tissue was subsequently embedded in OTC and stored at -80°C until acclimated and cryosectioned at -21°C into 20 µm thick sections and slide mounted.

RNA extraction

RNA was isolated using TRIzol (Invitrogen) per manufacturer's instructions. Briefly, the cells were lysed by direct application of TRIzol and five minute room temperature incubation. RNA was separated from the total lysate by addition of the chloroform. RNA was precipitated using isopropyl alcohol and ten minute room temperature incubation. Precipitated RNA was washed with 75% ethanol, and centrifuged to remove the ethanol. The final RNA pellet was redissolved in RNase free water and the concentration was measured using NanoDrop.

Protein Sample collection

To obtain protein lysates from cultured cells, I first suctioned off the media, rinsed the cells with PBS, and added 1X SDS sample buffer (22mM Tris-HCl pH 6.8, 4% Glycerol, 0.8% SDS, 1.6% β-Mercaptoethanol, Bromophenolblue; 200µl/well for 6 well plates, 2 ml/plate for 10cm plates) onto the cells. I collected the resulting gel-like lysed cells and immediately put them in the 95°C block for 5 minutes to unfold the proteins. The samples were kept in -20°C until ready to use for western blotting.

Western Gels

The SDS gels were made using the following chart:

Total (40 mL)	6% (mL)	10% (mL)	15% (mL)	Stack (mL)
DH ₂ O	21.1	15.8	9.2	17
30% Bis-Acrylamide	8	13.3	20	4.2
1.5M Tris-Cl (pH 8.8)	10	10	10	3.3 (pH 6.8)
10% SDS	.4	.4	.4	.25
10% APS	220 μ L	220 μ L	220 μ L	100 μ L
TEMED	40 μ L	40 μ L	40 μ L	20 μ L

Proteins larger than 100kDa were probed using the 6% gels, 50-100kDa proteins on 10% gels, smaller than 50kDa proteins on 15% gels.

Westerns

The boiled protein samples were separated by SDS-PAGE gels. I ran the protein at 80V until lysates clear the stack, and then increased the voltage to 120V. When the lysates complete the desired progression on the gel as indicated by their position relative to the ladder markings in the running buffer, I turned off the voltage and transferred the protein gel to a transfer apparatus. After transferring the proteins onto nitrocellulose membranes (Immobilon-P; Millipore) at 100V for 90 minutes, I blocked the resulting protein blot for one hour using 5% milk made in TBST (0.15 M NaCl, 20mM Tris, 0.05% Tween-20) buffer. Afterwards, I incubated the blots in primary antibody diluted in 5% milk or 5% BSA (for phospho-antibodies except pS6) overnight at 4°C. The next day, I replaced the primary

antibody solution with fresh TBST to wash at room temperature for ten minutes, three times, using fresh TBST each time. I then diluted the appropriate (depending on the species of the primary antibody) HRP-conjugated secondary antibodies in 5% milk at 1:1000 dilution. The blots were incubated in the secondary antibody solution on the shaker at low speed at room temperature for 2 hours. After, I washed the secondary antibody off with TBST, three times, ten minutes each. After the final wash, I incubated the blot in 1:1 volumes of luminol (250mM 3-aminophthalhydrazide, 90 mM p-coumaric acid in 100mM Tris, pH 8.6) and oxidizing solution (0.018% H₂O₂ in 100mM Tris pH 8.6) for three minutes. The resulting membrane was exposed on x-ray film (X-Omat AR, Eastman Kodak Co.) in the darkroom at varying lengths of exposure. Western blots were then quantified using ImageJ.

Immunohistochemistry

The slide-mounted sections were washed in phosphate buffered saline with Triton (PBST; 0.075M PBS; pH 7.30; 0.3% Triton; 0.9% NaCl) to permeabilize the membrane. After rinsing with PBS, the sections were incubated for one hour in 10% bovine serum albumin and 1% normal goat serum diluted in PBS as blocking step before overnight primary antibody incubation in 3% normal goat serum. After washing, the sections were incubated for 1 hour in Alexa-fluor 488 or 594 fluorescent secondary solution diluted at 1:1000 (Molecular Probes) and Hoechst 33342 solution (1:50,000) for DNA staining, washed three times for 10 minutes each before being coverslipped and imaged using Leica DM RXA microscope

equipped with epifluorescence.. For cell count statistics were performed in Prism5 using two-way ANOVA and student's t-test when otherwise noted.

Immunocytochemistry

Coverslips were collected into a light-protected hydration chamber. We fixed the cells using 4% PFA for ten minutes, and washed the coverslip three times, ten minutes each using fresh PBS. The cells were permeablized for ten minutes using phosphate buffered saline with Triton (PBST; 0.075M PBS; pH 7.30; 0.3% Triton; 0.9% NaCl). Following permeabilization, coverslips were incubated in blocking solution (5% NGS diluted in PBS) for 30 minutes. After blocking, I put on 100ul of diluted primary antibody solution per coverslip and incubated the chamber in the 4°C overnight. The following morning, I aspirated the primary antibody solution, washed the coverslip three times for ten minutes each with fresh PBS, and incubated coverslips with 100ul of fluorescence-conjugated Alexa-fluor 488 or 594 secondary antibody (Molecular Probes) solution at 1:500 and Hoescht 33342 solution for DNA staining at 1:50000 dilutions in PBS for one hour. After the hour, the coverslips were washed three times for ten minutes each then mounted onto a slide using fluoromount media, and kept in 4°C until imaged. Images were then viewed with a Leica DM RXA microscope equipped with epifluorescence.

Antibody

The following antibodies were used: PDGF-R α (mouse; BD Pharminogen), O4 (Rabbit; Millipore), A2B5 (mouse; Millipore), CTGF (goat; Santa Cruz), pS6 (rabbit;

Cell Signaling), Cleaved Caspase-3 (Rabbit; Cell Signaling), AKT (Rabbit; Cell Signaling), TSC2 (Rabbit; Santa Cruz), MBP (Rabbit; Millipore), MAG (mouse; Sigma), PMP70 (Rabbit; Abcam), Catalase (Rabbit; Abcam), Tau1 (mouse; Millipore).

EM images and sample collection

Four week old adult mutant (*Tsc1^{flox/flox}Syn1Cre⁺*) and control (*Tsc1^{flox/+}Syn1Cre⁺*) mice were perfused intracardially using 1.25% PFA, 2.5% glutaraldehyde, and 0.03% picric acid in 100 mM cacodylate buffer for EM images. We analyzed untreated and rapamycin-treated animals in each genotype, totaling 11 animals, and at least 200 axons per animals. The brain was removed and post fixed over night. The medial corpus callosum was identified from vibratomed brain coronal sections (350-μm thick) and embedded in resin. At least thirty non-overlapping regions from each animal were photographed at 20,000X magnification. In order to calculate the g-ratio, we used a tablet digitizer (SummaSketch III Summagraphics, Seattle, WA) and ImageJ software. The g-ratio was calculated for each myelinated axon as axon diameter/myelin sheath diameter by a researcher blind to the genotypes and treatment conditions. We also used the images to count the number of unmyelinated axons as well as calculate their axon diameter. The data across the four experimental groups were compared using ANOVA.

Cortical Neuron Cultures

I collected the conditioned media from cultured cortical neurons as previously published from our laboratory (Sahin et al., 2005). Briefly, the cortices

from embryonic day 18 rats (Charles River CD1) were isolated under the microscope and collected in Hank's Balanced Salt Solution containing 10 mM MgCl₂, 1mM Kynurenic acid, 10 mM HEPES, and penicillin/streptomycin. The cortices were dissociated in 30 u/ml papain at 37°C for five minutes, and mechanically triturated and plated in Neurobasal medium (Invitrogen) containing B27 supplement, 2 mM L-glutamine, and penicillin/streptomycin (Invitrogen). For medium collection, the cells were plated at 1 x 10⁷ cells/10cm plate coated with poly-D-lysine. For CTGF regulation experiments, we used UO126 at 20 μM, CI-1040 at 10nM, LY294002 50 μM and rapamycin at 20 nM overnight.

Lentivirus Production and Infection

After two days of recovery and medium change the neurons were infected with lentivirus against either *Tsc2* or *Gl3* (control shRNAi construct against the luciferase gene; Flavell et al., 2006). The virus was made as previously published (Mostoslavsky et al., 2005; Di Nardo et al., 2009). Briefly, the four packaging vectors (generous gift of Dr. R.C. Mulligan) were co-transfected into HEK293T cells with either the *Tsc2*-shRNAi or *GL3*-shRNAi plasmids using Lipofectamine 2000 according to the manufacturer's instructions. The resulting viral particles were collected 48 and 72 hours after the transfection and filtered through the 0.45μm membrane, and the aliquots were stored in -80°C until usage.

The cortical neurons were infected at 2 days in vitro in the presence of (0.6 ug/ml) polybrene. Six hours after infection, the virus was washed out with neurobasal and the medium was replaced with NB/B27. After infection, the neurons

were kept in culture for 10 more days for 90% knockdown efficiency of the *Tsc2* gene. The *Tsc2* targeting sequence (Invitrogen) was: 5'-GGTGAAGAGAGCCGTATCACA-3'.

Conditioned media collection and cell treatment

Seven days after the lentivirus infection, I completely replaced the neuronal media with 5 ml of fresh NB/B27 media per 10cm plate. Four days later, the media was collected, placed into pre-hydrated Vivaspin concentrator tubes (Sartorius), and immediately centrifuged for 35 minutes at 3400 RPM at 4°C. This allows for 10x concentration of volume of media. The aliquots of this media were stored at -20C until use. To inhibit CTGF function, neutralizing antibody to CTGF (Santa Cruz) was added to the media at 100 µg/ml and incubated at 4°C on the rocker for one hour. For conditioned media treatment experiments, the concentrated media was mixed 1:1 with normal OL media (1 mM sodium pyruvate, 25 µg/mL gentamycin, 5 µg/mL insulin, 50 µg/mL transferrin, 30 nM selenium, 30 nM triiodo-thyronine, 1% v/v L-glutamine, and 0.5% FBS in DMEM) and placed on the cells immediately following mitogenic de-differentiation of the cells to OPCs.

OL cultures

I performed OPC purification cultures from mixed cortical glial cultures using established methods (McCarthy and de Vellis, 1980). I removed the cortices of P1 Sprague Dawley rat pups, excluding the hippocampus, meninges, and the basal ganglia. Cortices were mechanically dissociated using 22 gauge and 25 gauge

needles. The dissociated cortices were filtered through the 70µm cell strainer and spun down before being plated on poly-D-lysine coated T75 flasks in 10% FBS, 2mM L-glutamine, 25 µg/mL gentamicin in Dulbecco's minimum essential media (DMEM). I maintained the culture in 10% CO2 humidified incubator for seven to ten days, changing the media every three days.

The OPCs were purified using the differential shake method. Briefly, after seven to ten days, the T75 flasks of mixed glial cultures were shaken at 180 RPM for one hour to remove the microglia, and the fresh media (10% FBS) was added before the flasks were shaken overnight in humidified, 37°C incubator. Next day, the resulting supernatant was filtered through 40-µm mesh filter, and spun down, reconstituted in 8 ml media and seeded on uncoated 10cm tissue culture dish for one hour to allow any remaining fibroblasts to stick. The floating OLs were collected after the hour, and seeded onto poly-D-lysine and mouse-laminin coated glass coverslips in chemically defined media consisted of 1mM sodium pyruvate, 25 µg/mL gentamycin, 5 µg/mL insulin, 50 µg/mL transferrin, 30nM selenium, 30nM triiodo-thyronine, 1% v/v L-glutamine, and 0.5% FBS in DMEM. The OPCs were seeded at 10,000/coverslip and allowed recover and amplify in the presence of 10 ng/mL each of PDGF and b-FGF for three days before differentiation.

The amplification and synchronization of the purified OL cultures to PDGF-R α + /NG2+ OPCs was a critical step in the identification of the function of CTGF. As I was the first person to establish the OPC culture system in our laboratory, I learned through my experiments the necessity of this step. Upon treating the cultures with the neuronal conditioned media (as described above and in the next chapter), I

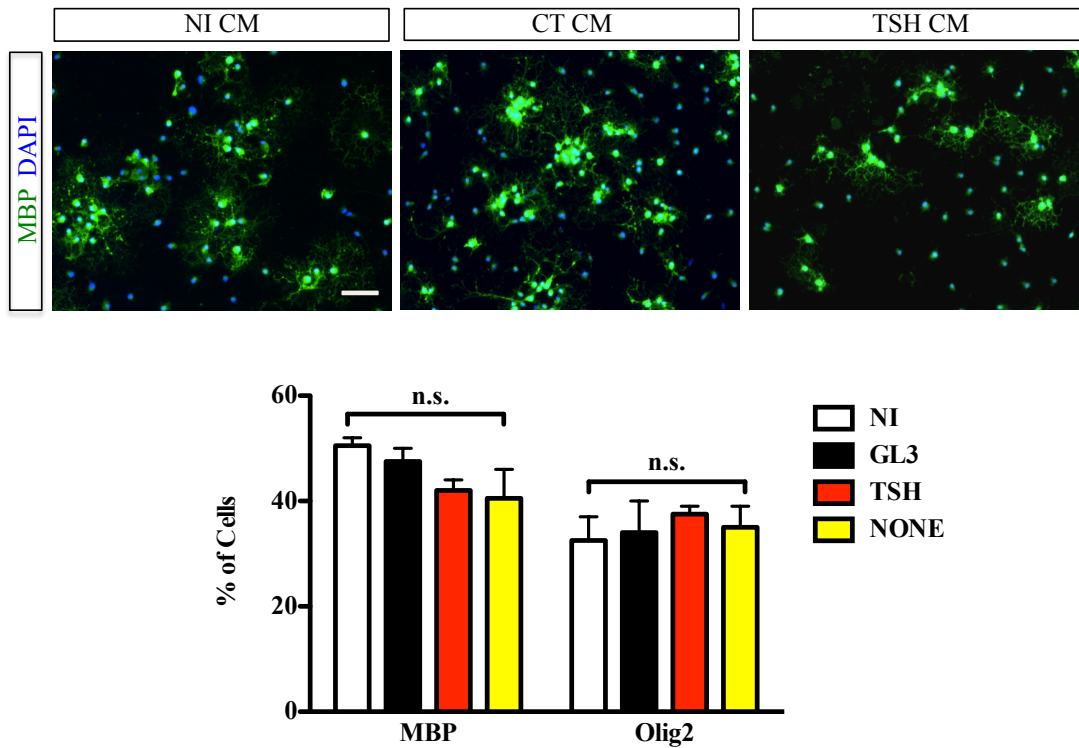


Figure 2.1. Without growth factor amplification and synchronization of the OPC lineage, there is no consistent quantitative effect of the experimental conditioned media despite qualitative differences observed in the morphologies of the MBP-positive cells. scale bar = 100 μ m

found inconsistencies across the same experimental groups despite the same conditions, resulting in statistically insignificant data (Figure 2.1).

ELISA (Enzyme-linked immunosorbent assay)

CTGF concentrations in the conditioned media were measured using the ELISA kit (Peprotech, USA). I first coated the microplates (Nunc MaxiSorp) with 1µg/ml CTGF capture antibody overnight at room temperature. The wells were then blocked with 1% BSA in PBS (50mM Tris pH 7.4, 0.8% NaCl, 0.02% KCl) for one hour at room temperature. The human recombinant CTGF was loaded as standards in triplicate from 4ng/ml to zero concentration. The sample condition media was also loaded in triplicate and incubated in the wells at room temperature for two hours. The wells were then washed with 0.05% Tween PBS four times, and detection antibody was added onto the wells at 0.5 µg/ml. After two hours of incubation, avidin-HRP conjugate solution was added into the wells. To visualize the reaction, ABTS liquid substrate (Sigma) was added into the wells and the plates were read at 405nm.

Microarray

For the microarray experiments, the cortical neurons were plated into the 3 um Boyden Chamber inserts at 1M cells/insert in 1.5ml media (NB/B27) on top of the chamber and 1.5 ml of media (NB/B27, BDNF 100 ng/ml (Peprotech) to stimulate growth of the neurites toward the bottom chamber) on the bottom. 24 hours after plating, the neurons were infected with the TSC2 or GL3 KD virus as

described above for six hours. Fresh media was provided after three days including the BDNF to the bottom chamber until 11 days after infection. On the 11th day post infection, top (Soma) and bottom (neurites) cell materials were collected by gentle scalpel scraping. The scraped cell materials were spun down in the cold room for fifteen minutes until prepared for microarray. We then performed gene expression profiling using Illumina micro-arrays in collaboration with Dr. Dan Geschwind (UCLA).

The analysis of the microarray was performed by our collaborator Dr. Dennis Wall. Briefly, we imported the Illumina beadchip microarray data into R/Bioconductor for normalization, quality control and detection of differential expression. We used the beadarray library from Bioconductor to process the raw expression data (Dunning et al., 2008). We then performed quantile normalization to control for chip-specific variation in expression. To detect differential expression we used the functions `lmfit` and `ebayes` from the `limma` package after performing a \log_2 data transformation on the normalized expression matrix. We then set up a design matrix to generate contrasts between control and TSC cell body and axon and perform an empirical bayes analysis to statistically control the differential expression magnitudes. Finally, we used bonferroni correction to control for multiple testing.

Plasmids

The plasmids used in Chapter 5 were obtained from the laboratory of Dr. Cheryl Walker. Briefly, the flag-TSC2 plasmids were described previously (Cai et al.,

2006). The TSC2 mutation plasmids harboring mutations in a.a. 1743 G, Q, W were generated using site-directed mutagenesis by using Staratagene QuikChange Kit and were validated by sequencing.

RT-PCR

For reverse transcription, I used 2 µg poly(A) mRNA and High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) per manufacturer instructions. Briefly, the mRNA was mixed with RT buffer, dNTP, 10X random primers, reverse transcriptase, and nuclease free water. The tubes were processed through the thermal cycler using the following conditions: 25°C 10 minutes, 37°C 120 minutes, 85°C 5 seconds. The resulting cDNA strands were stored in 4°C.

For quantitative polymerase chain reaction, I used the SYBR green kit (Invitrogen) in triplicate for each sample and normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control for each sample. The qPCR cycle was: 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for one minutes. Analysis was performed using 7300 System SDS Software on a 7300 Real Time PCR System. The data was expressed as means \pm SE of at least three independent experiments. Statistical analysis was performed by unpaired two-tailed Student's t test and considered significant at $p < 0.05$.

Primers

Gene	Primer
Tsc2	F: 5' GAGCTGTGTGACCAGAACGA-3'
	R: 5'-TGTTGATCAGCAGCACGAAG-3'
Mbp	F: 5'-ATCCAAGTACCTGGCCACAG-3'
	R: 5'-CCTGTCACCGCTAAAGAAGC-3'
Plp	F: 5'-GAGGCCAACATCAAGCTCAT-3'
	R: 5'-GACTGACAGGTGGTCCAGGT-3'
Cnp	F: 5'-AGCTCAAGGAGAAGAACCAAT-3'
	R: 5'-TGAAGTGTCGAAGCTCTTTCT-3'
Ho-1	F: 5'-TGCTCGCATGAACACTCTG-3'
	R: 5'-TCCTCTGTCAGCAGTGCCT-3'
ctgf	F: 5'-AGCAGCTGGGAGAACTGTGT-3'
	R: 5'-GCTGCTTTGGAAGGACTCAC-3'
gapdh	F: 5'-TGTGTCCGTCGTGGATCTGA-3'
	R: 5'-CCTGCTTCACCACCTTCTTGA-3'

Chapter 3

The Role of Neuronal TSC/mTOR in Oligodendrocyte Maturation

Summary

TSC is an autosomal dominant syndrome characterized by many neurodevelopmental abnormalities. Until recently, research in the field has focused on tuber formation as well as neurons and astrocytes. However, growing evidence in TSC patients as well as *Tsc* mouse models shows that white matter deficits play important roles in the manifestations of TSC. We have previously shown that TSC1 deficiency in neurons is sufficient to give rise to developmental hypomyelination, but the underlying mechanism has not been previously identified. Therefore, we took a novel research perspective to investigate the role of TSC-mTOR pathway in axon-oligodendrocyte interaction. Here we show that the hypomyelination deficit in the neuronal *Tsc1*-knockout mouse model comes from arrested differentiation of the oligodendrocytes rather than a primary deficit in the amount of myelin produced by individual cells. We used a genome-wide gene expression analysis in TSC-deficient cultured primary neurons and identified connective tissue growth factor (CTGF) as a potential paracrine signaling molecule regulating oligodendrocyte differentiation. We confirmed that this factor was upregulated in

Tsc2-deficient neurons *in vivo* and *in vitro*. Furthermore, we demonstrated that this factor was sufficient to inhibit the differentiation of the oligodendrocytes precursor cells (OPCs) *in vitro*, and that inhibition of CTGF function by immunological method can prevent the differentiation arrest of conditioned media from *Tsc2*-deficient neurons. Finally, we show that CTGF is expressed in TSC-deficient pS6 positive cells of the TSC patient brains. Together, these studies provide the first mechanistic evidence of neuronal TSC/mTOR modulation of oligodendrocyte maturation, and highlight the therapeutic potential for targeting CTGF.

Introduction

Tuberous Sclerosis Complex (TSC) is an autosomal dominant multisystem disorder where 90-95% of patients present neurological manifestations such as epilepsy, behavioral abnormalities, intellectual disability, and autism (Crino et al., 2004). TSC patients present with abnormalities in their white matter both focally underlying tubers but also more diffusely in major intrahemispheric tracts, resulting in approximately 15% reduction in white matter volume (Ridler et al., 2007). Past CNS research in the field has focused on formation of cortical tubers and epilepsy based on the theory that these abnormalities directly contribute to the cognitive deficits and seizure episodes. However, the recent studies provide conflicting evidence regarding link between tubers and clinical severity. In fact, hypomyelination and aberrant axon guidance are characteristics of CNS development that can alter proper synaptic function and connectivity that correlate best with the severity of clinical disease.

TSC is caused by mutation in either *TSC1* or *TSC2* gene that encode for hamartin and tuberlin, respectively. These two proteins bind each other to form a complex that plays a critical role in the protein synthesis regulation pathway, which controls cell growth and size in response to growth factor stimulation (Kwiatkowski and Manning, 2005). One of the functions of the TSC complex is to inhibit Ras family GTPase, Rheb, whose downstream targets include serine-threonine kinase mammalian target of rapamycin (mTOR), a master regulator of protein synthesis. Thus, without functional TSC complex, mTOR is hyperactive, resulting in disinhibited protein synthesis and subsequent cell growth. In addition, TSC deficiency has been found to cause aberrant migration that leads to cortical disorganization, altered proliferation and differentiation, implicating its role in modulating cell cycle and fate determination.

We have previously demonstrated that the neuron-specific conditional *Tsc1* knockout results in disrupted myelination as evidenced by reduced staining for myelin basic protein (MBP; Meikle et al., 2007). Postnatal rapamycin drastically improved MBP staining, indicating that the myelination development mechanism in these mice was dependent on neuronal mTOR activity (Meikle et al., 2008). Although the oligodendritic mTOR has been implicated as a critical regulator of both oligodendrocyte differentiation and myelination, it is not yet clear how neuronal mTOR activity can alter the neuron-oligodendrocyte interactions (Narayanan et al., 2009; Tyler et al., 2009).

Oligodendrocyte development is a carefully orchestrated event, involving multiple intrinsic and extrinsic factors. Although they have the ability to

spontaneously differentiate, oligodendrocytes are responsive to extrinsic cues and activities of the surrounding cells such as neurons and astrocytes. Although a number of factors such as secreted IGF, electric activity of the neurons (Stevens et al., 2002), and membrane-bound ligands (e.g. Neuregulin/ErbB3, Notch-Jagged/delta) have been implicated in oligodendrocyte maturation in normally developing brains, it is not yet clear how diseased neurons such as those in the TSC mouse models can directly inhibit their proper development.

Here we show that genetically normal oligodendrocytes differentiation is arrested in the presence of Tsc-deficient neurons *in vivo*. Treating the OPCs with conditioned media collected from Tsc-knockdown neurons was sufficient to mimic this phenomenon *in vitro*, indicating that anomalous paracrine signaling from the neurons was responsible for inhibiting differentiation of the oligodendrocytes. Using genome-wide expression profiling we identified connective tissue growth factor (CTGF) as an upregulated signaling molecule that is expressed near the corpus callosum during the time of oligodendrocytes differentiation. Application of CTGF alone was able to reduce the differentiation of the OPCs while neutralization and depletion of CTGF was able to rescue the number of oligodendrocytes that express MBP. We also found that CTGF is upregulated in Tsc-deficient cells of the brain, alluding to its potential as a therapeutic target.

Results

We first wanted to characterize the timing and extent of hypomyelination in the mutant mice to isolate the source of hypomyelination. Because our prior study

used reduced MBP as marker of reduced myelin, it was unclear whether this reduction in expression of the myelin protein marker was due to decreased number of oligodendrocytes expressing MBP or because the myelinating sheaths are thin. To address this question, we performed EM analysis on the corpus callosum of mutant and control mice treated with rapamycin or vehicle at dose and frequency previously shown to be effective in reversing the hypomyelination in the mutants. We chose to study the corpus callosum, given that it is the largest white matter tract in the brain and that it has been severely affected by neuronal Tsc deficiency in mice as well as in TSC patients (Meikle et al., 2007; Peters et al., 2012). Qualitatively, the corpus callosum axons of the untreated mutants were extremely enlarged and appeared disorganized; Figure 3.1). Quantitatively, the diameters of the myelinated axons were increased (Figure 3.2; control = 0.55 ± 0.01 , $n = 497$; mutant = 0.75 ± 0.02 , $n = 173$; $p < 0.0001$), and this increase was reversible with rapamycin treatment; in the mutants, the diameters of the myelinated axons decreased (Figure 3.2; Control + Rapa = 0.56 ± 0.01 , $n = 673$; mutant + Rapa = 0.49 ± 0.01 , $n = 262$; $p < 0.0001$ between mutant untreated and mutant treated groups). Interestingly, the rapamycin treatment had no effect on the control group diameter size ($p = 0.42$, between control untreated and control rapamycin treated), despite mutant axons becoming significantly smaller than the control treated group ($p < 0.0001$).

We next investigated the thickness of myelin by measuring the g-ratios of the four experimental groups. G-ratio is a numerical value reflecting the relative thickness of the myelin, attained by dividing the axon diameter by the total fiber diameter, which is inclusive of axon and the myelin sheath. This figure therefore

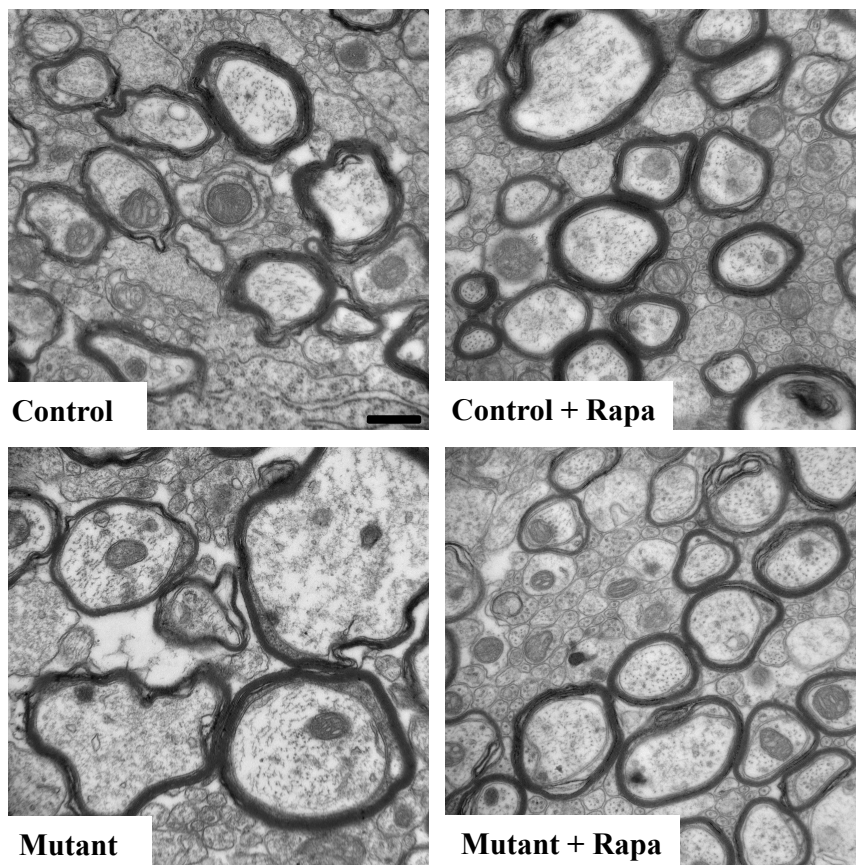


Figure 3.1. Representative electron microscopic (EM) images of mutant and control mice corpus callosum, treated or untreated with rapamycin from P7. Without treatment, the mutant axons are enlarged and disorganized. Scale bar = 500 μ m

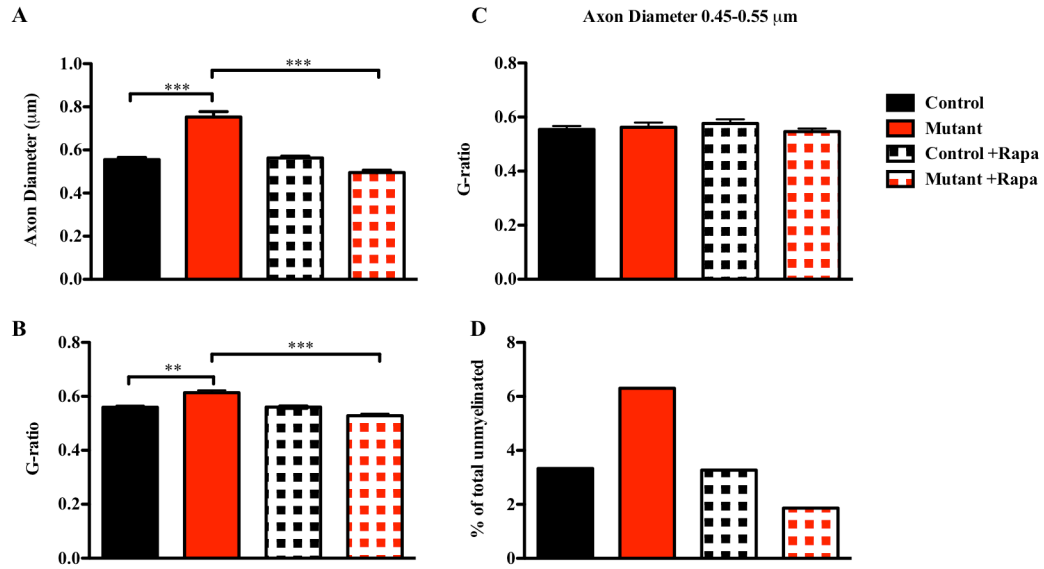


Figure 3.2. **A.** The axon diameter of the untreated mutant group was significantly increased (control, 0.55 ± 0.01 , $n = 497$; mutant = 0.75 ± 0.02 , $n = 173$). This increase was reversible by rapamycin treatment (control Tx, 0.56 ± 0.01 , $n = 673$; mutant Tx, 0.49 ± 0.01 , $n = 262$). **B.** The g-ratio of the untreated mutant group was increased, indicating relatively thinner myelin (control, 0.61 ± 0.008 vs. mutant = 0.56 ± 0.006). Rapamycin treatment inhibited this increase in the mutants but had no effect on the control group g-ratio (treated control, 0.56 ± 0.01 vs. mutant g-ratio, 0.53 ± 0.01). **C.** The g-ratio across equal diameter axons were not different across the groups. **D.** The percentage of unmyelinated axons within the $0.45 \mu\text{m}$ - $0.55 \mu\text{m}$ were increased in the untreated mutant group. (***) $p < 0.0001$; ** $p < 0.001$)

takes into account the size of the axon as well as the thickness of the myelin; the thicker the myelin wrapping the smaller the g-ratio. The untreated mutant group had a significantly higher g-ratio than the control untreated group (Figure 3.2; 0.61 ± 0.008 vs. 0.56 ± 0.006 ; $p < 0.001$), indicative of thinner myelin relative to the axon size. This difference was rescued by rapamycin treatment while the same treatment had no effect on the control animals (0.56 ± 0.01 vs. 0.53 ± 0.01). G-ratio is a widely accepted measure of myelin thickness, which tightly correlates with an increase in axon size. However, since the *Tsc1*-deficient axons are so enlarged, it may be possible that the genetically normal oligodendrocytes have simply reached the maximal number of myelin wraps they are able to make. If so, G-ratio would not properly reflect this phenomenon. To begin to address this possibility, we first counted the number of layers of myelin sheath and found no significant differences between the groups or notice any differences in packing of the layers across experimental groups (data not shown). In fact, when the g-ratio was compared between the same sized axons (0.44-0.55 μm size group where most axons should be myelinated), there was no difference among the experimental groups, further reinforcing the notion that the decrease in myelination may arise from a different source than having individually thinner myelin alone. In fact, we saw an increase in the percentage of unmyelinated axons within this diameter size range in the untreated mutant group, suggesting that the overall lowered MBP levels in the mutants may result from a decrease in the number of oligodendrocytes rather than the amount of myelin produced per oligodendrocyte. The size of unmyelinated axons was unchanged in the untreated mutants, indicating that despite all neurons

being deficient of *Tsc1*, the myelinated large diameter axons are somehow differentially, possibly even more severely, affected by the loss of *Tsc1*.

Because oligodendrocyte development occurs in distinct stages, we next wanted to investigate more closely the number and morphology of oligodendrocytes in *Tsc1^{flox/flox}SynICre* mice, and look into the possibilities of arrest in differentiation, loss of branching, and cell death as the sources of hypomyelination. To better visualize the number and distribution of oligodendrocytes in the mice, we crossed the *Tsc1^{flox/flox}SynICre* mice to a mouse strain that expresses enhanced green fluorescent protein (eGFP) under the control of the proteolipid protein (Plp) promoter thereby allowing us to label oligodendrocytes (Murtie et al., 2007). Genetic expression of EGFP in myelinating oligodendrocytes allowed us to quantify the number of PLP-expressing cells with high accuracy. In *Tsc1^{flox/flox}SynICre⁺;Plp-eGFP⁺* mice, we found the density of EGFP⁺ oligodendrocytes to be markedly decreased in the corpus callosum by a factor of two as compared with control mice at P21 (Figure 3.3, 3.4; control = 210 ± 8.8 cells/ 0.1mm^2 vs mutant = 113.3 ± 30 / 0.1mm^2 ; $p < 0.0005$). We also observed a similarly drastic decrease in the number of EGFP⁺ cells in the grey matter of the mutant mice (control = 24.1 ± 1.9 cells/ 0.1mm^2 vs mutant = 8.2 ± 0.49 / 0.1mm^2 ; $p < 0.0005$). Sparse white matter area occupied by the oligodendrocytes made the interhemispheric tract appear significantly smaller. To see whether the gross anatomy of the area surrounding the corpus callosum was reduced, we stained the sections with DAPI to visualize the overall cell number and distribution and found no such anatomical differences (data not shown). In fact, our previous study has shown that these mice do not appear to

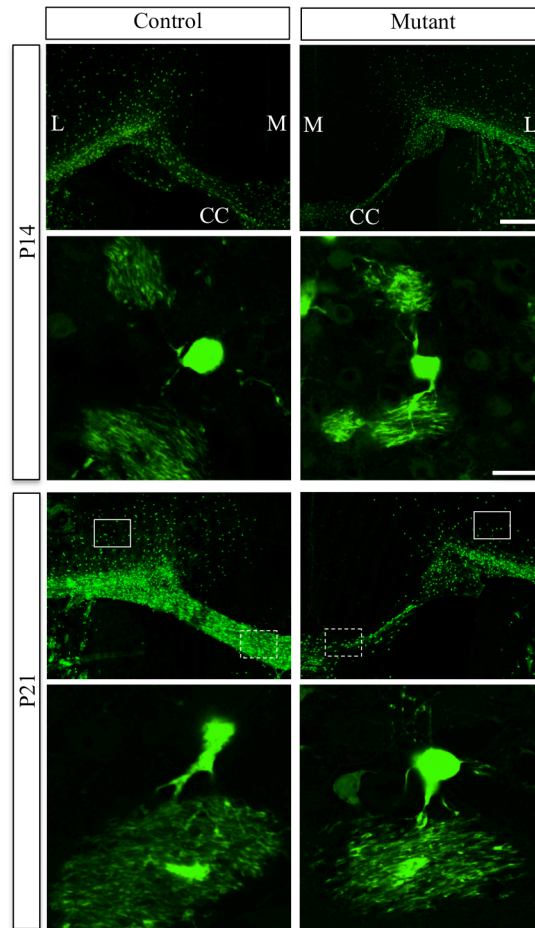


Figure 3.3. At P14, Plp-eGFP mutant and control mice show no significant differences in the number of eGFP+ oligodendrocytes in the corpus callosum. Morphology of the individual plp+ oligodendrocyte looks indistinguishable. L=lateral, M=medial, CC=corpus callosum At P21, there was a dramatic increase in eGFP+ cells in the control brains that was not seen in the mutant brains. The boxed grey matter and dash boxed white matter corpus callosum areas are quantified in figure 3.4. The morphology of myelinating oligodendrocyte was still not different between the two groups. Scale bar in corpus callosum images = 400 μ m, cell images = 20 μ m

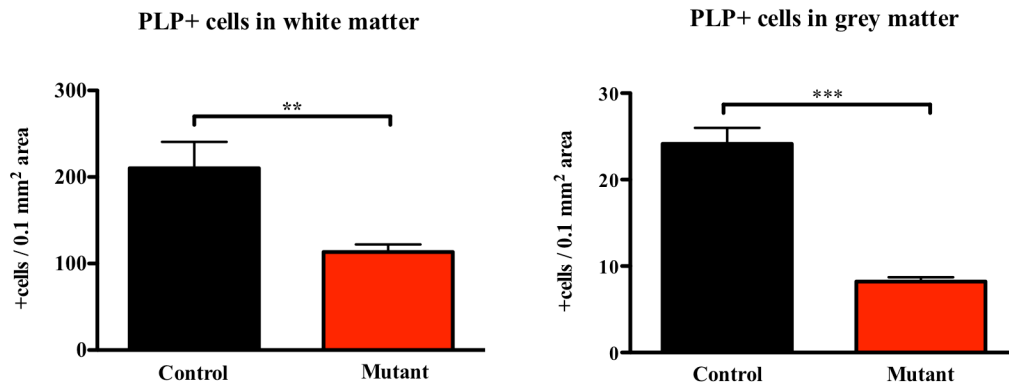


Figure 3.4. The number of eGFP+ cells in the corpus callosum as sampled from dashed box in the image was decreased in the P21 mutant brains (control, 210 ± 8.8 cells/ 0.1 mm^2 vs mutant, $113.3 \pm 30 / 0.1 \text{ mm}^2$; $p < 0.005$). Similar decrease was seen in the grey matter of the mutants (control, 24.1 ± 1.9 cells/ 0.1 mm^2 vs. mutant, $8.2 \pm 0.49 / 0.1 \text{ mm}^2$; $p < 0.0005$).

have gross axonal projection deficits in the cortical area (Meikle et al., 2007). This difference in EGFP+ cells was not visually present in the P7 cortices and was not significantly different in P14 corpus callosum between the mutant and the control, suggesting that between P14 to P21, either oligodendrocytes had failed to differentiate or died. It also demonstrated that the hypomyelination was a result of dysmyelination mechanisms, not due to degenerative demyelination.

Using confocal microscopy, we next closely examined the morphology of the oligodendrocytes to determine whether the ones that are present and myelinating are normal. We found no gross morphological (size, shape, branching pattern) difference as observed by confocal microscopy (Figure 3.3).

We next asked whether the decrease in the number of oligodendrocytes in the mutant mice could be due to apoptosis during development. We immunostained the brain sections at P7, P14, and P21 for cleaved caspase-3, a marker for apoptosis and found no substantial cell death in the control or the mutant mice, indicating that decreased oligodendrocyte number was not likely due to apoptosis.

We next hypothesized that the hypomyelination was a result of shortage in mature oligodendrocytes due to arrest in differentiation or lack of proliferation. To test this hypothesis, we immunostained the control and mutant brain sections with A2B5 and PDGF-R α at P7 and found no differences in the number of proliferating cells, indicating that the shortage in the number of oligodendrocytes arise most likely after this stage (Figure 3.5). We next stained the P7, P14, and P21 sections for O4 and olig2, markers of postmitotic immature oligodendrocytes. We saw that although their numbers were similar across the groups at P7, the immature

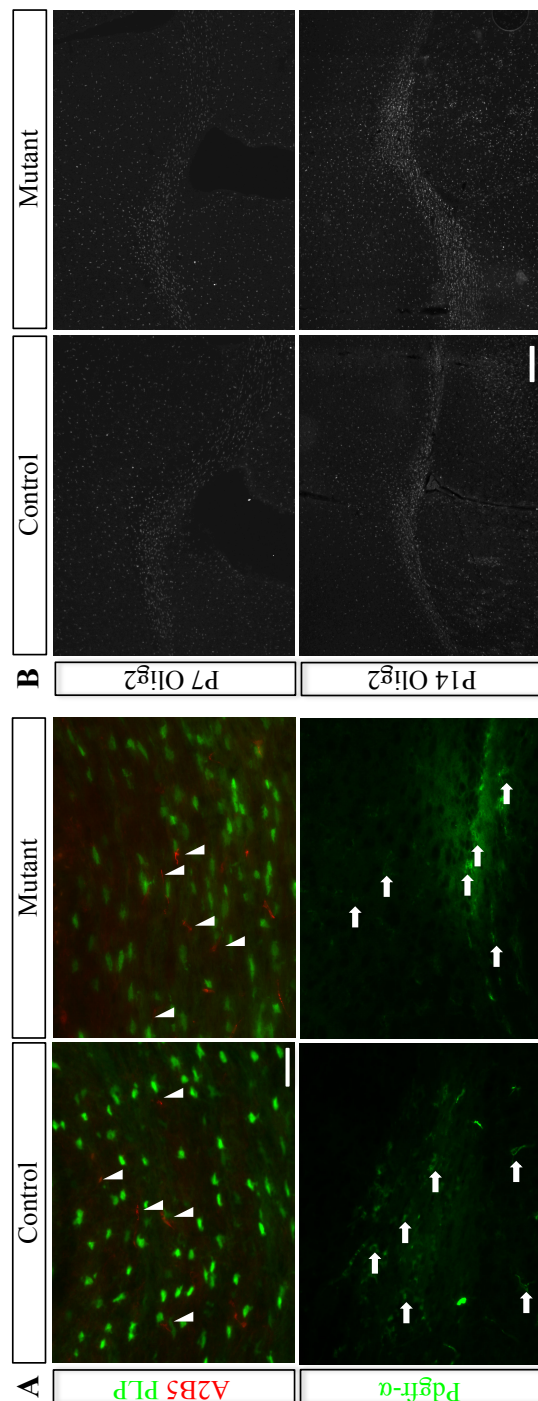
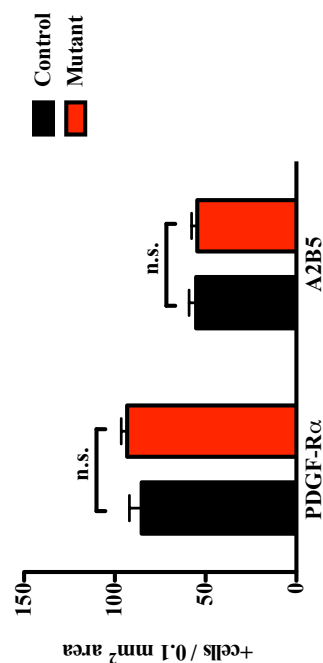


Figure 3.5. A. OPCs in the corpus callosum were stained with A2B5 and Pdgfr- α at P7 in the mutant and control brains. There was no significant differences in the cells numbers between the two experimental groups (PDGF-R α + cells control $85.33 \pm 6.7/0.1\text{mm}^2$ vs mutant 93.3 ± 3.3 0.1mm^2 ; A2B5+ cells $55 \pm 3.8/0.1\text{mm}^2$ vs mutant $54.7 \pm 3.2/0.1\text{mm}^2$). Scale bar = $100\text{ }\mu\text{m}$ **B.** There was no difference in Olig2 positive cells at P7, but P14 brains of the mutant continued to increase the Olig2 positive cells. Olig2 positive cells did not co-stain with Plp-eGFP oligodendrocytes. Scale Bar = $250\text{ }\mu\text{m}$



oligodendrocyte numbers were strikingly higher by P14 and the cells continued to express O4 and olig2 beyond this age, indicating a block in differentiation of the oligodendrocytes at this stage (Figure 3.5; O4 not shown).

Since the oligodendrocytes in these mutants are genetically normal, we hypothesized that a neuron-generated molecule regulates the differentiation of the oligodendrocytes, and that the molecule was dysregulated in *Tsc*-deficient neurons. To test if a secreted neuronal factor was necessary for regulating oligodendrocyte differentiation, we treated the A2B5+/PDGFRa+ OPCs with conditioned medium (CM) collected from control and *Tsc2*-deficient cortical neurons. Although our mice were deficient of *Tsc1*, *Tsc2* deficiency produces the same results in vitro given that the proteins were obligatory for each other. We found that after three days of treatment of OPCs, *Tsc2*-knockdown CM markedly reduced the number of MBP-positive cells compared with control (CT) CM treatment (Figure 3.6; CT CM, $98.72 \pm 2.6\%$; KD CM, $14 \pm 1.2\%$; normalized to non-infected control treatment condition). We then asked whether this was due to block in differentiation of the oligodendrocytes and indeed, similar to our *in vivo* data, a significant number of *Tsc2*-KD CM treated OPCs failed to differentiate beyond the O4+ stage. Neither conditioned media treated culture resulted in differences in cell death as indicated by cleaved caspase 3 staining (Figure 3.8). Together, this data indicated that a secreted factor, either excessive inhibitory or decreased permissive/instructive cue that can modulate the oligodendrocyte differentiation was differentially regulated in *Tsc*-deficient neurons.

To identify the neuronal signals that regulate myelination, we performed a microarray analysis of neurons with and without *Tsc2* expression. We knocked down *Tsc2* using lentivirus shRNA (TSH) in modified Boyden chambers and extracted mRNA from the cell body or the neurite compartment of the neurons. We then performed gene expression profiling using Illumina microarrays in collaboration with Dr. Dan Geschwind (UCLA). Among the genes differentially expressed in *Tsc2*-deficient neurons, we focused on those encoding secreted proteins, expressed in early postnatal ages and previously implicated in oligodendrocyte differentiation. Only connective tissue growth factor (CTGF) fulfilled all these criteria. Our microarray data showed that CTGF transcription was significantly upregulated in TSC2-deficient neurons in both cell body and neurite compartments (adjusted p-value 0.0036 based on six biological replicates).

To confirm the increase in CTGF transcripts, we performed qRT-PCR from purified RNA from TSC2-knockdown (TSH) neurons and found 1.5-fold increase in the transcripts (Figure 3.7). We then performed westerns to test whether the increase in transcripts led to increase in protein. There was also a significant increase in the CTGF protein in the knockdown neurons compared to the control neurons, and this increase was blocked by 20 nM rapamycin treatment for 12 hours, indicating that the upregulation of this protein was mTORC1 dependent (Figure 3.7). We next asked whether CTGF is secreted *in vitro* and whether increase in the protein translated resulted in increased CTGF secreted into the conditioned media. Using an ELISA assay, we found that the amount of secreted CTGF was also significantly increased in neuronal CM (Figure 3.7). These data shows that CTGF

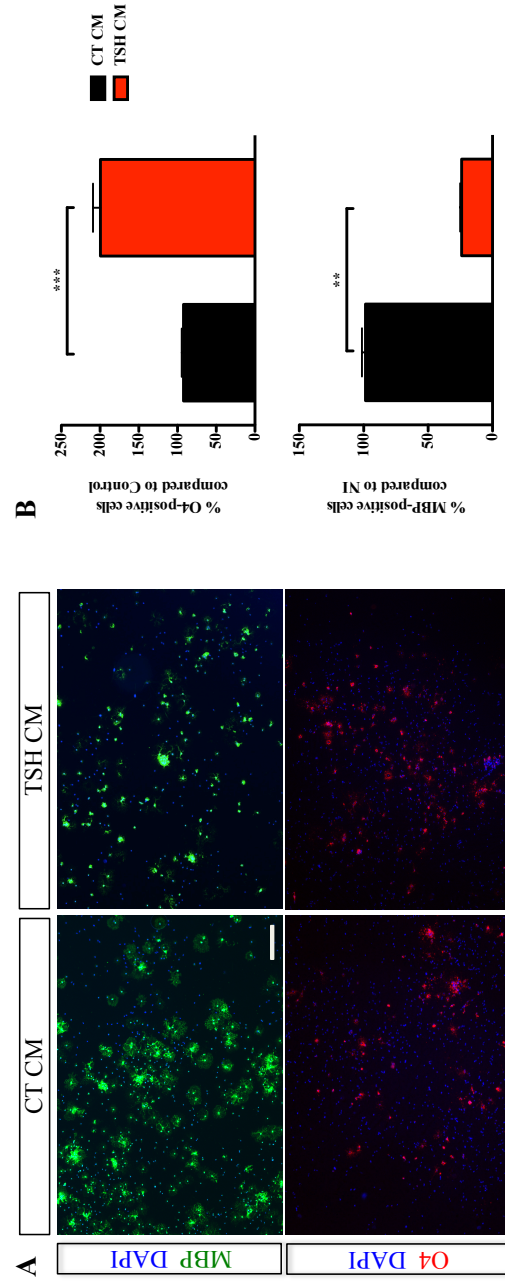


Figure 3.6. TSH CM (CM from neurons treated with Tsc2-knockdown lentivirus) treated oligodendrocytes show reduction in differentiation. **A.** Conditioned media collected from control knockdown (CT CM) or Tsc2-knockdown (TSH CM) were added onto OPC cultures and stained for MBP or O4. scale bar = 100 μ m **B.** TSH CM treated cells show higher number of O4 positive cells and lower number of MBP positive cells (CT CM, 92.33 ± 2.4 ; TSH CM, 199.7 ± 9.7). ** $p < 0.001$; *** $p < 0.0001$

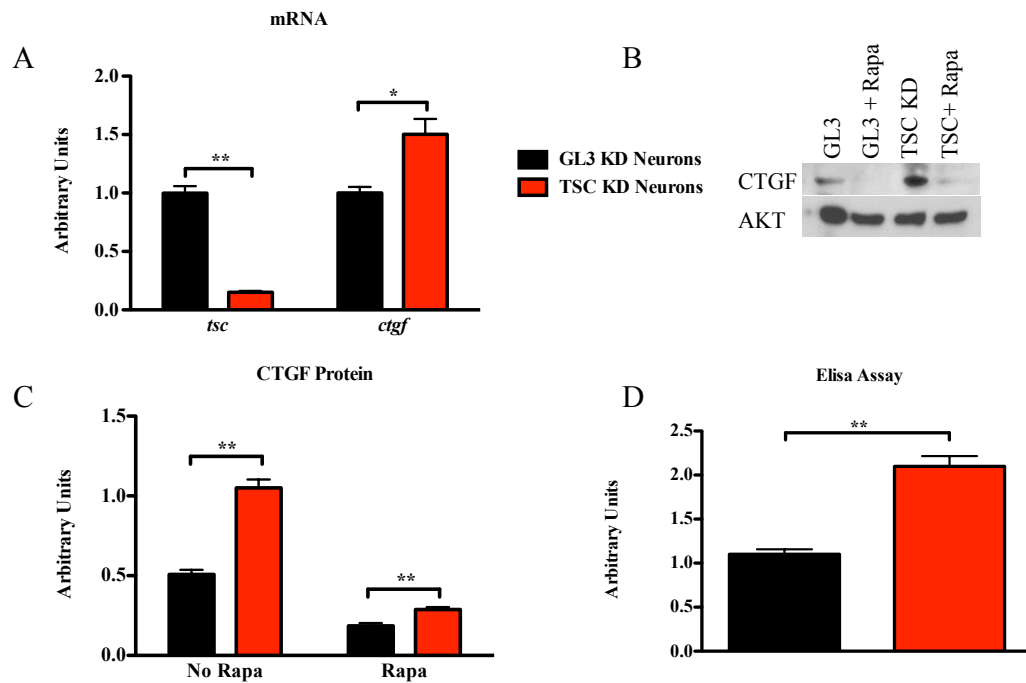


Figure 3.7. CTGF is upregulated in vitro. **A.** *Ctgf* mRNA transcripts are upregulated when neurons lose approximately 80% Tsc2. **B.** representative western blot showing upregulation of CTGF protein in neurons with Tsc2 knockdown. Overnight rapamycin treatment can reverse this increase showing mTOR-dependency of CTGF regulation. **C.** Three independent western blot experiments were quantified to show the upregulation in Tsc2-deficient neurons and reversal with rapamycin treatment. **D.** Secreted CTGF is also significantly upregulated in the Tsc2-deficient neuron media.

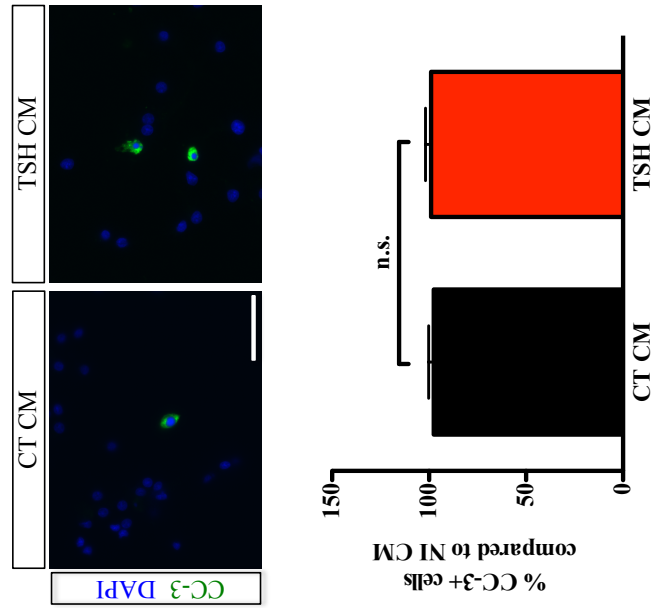


Figure 3.8. There was no significant differences in the number of cleaved caspase-3 positive cells in the control conditioned media treated oligodendrocyte culture compared to the Tsc-knockdown neuron conditioned media culture. Scale bar = 100 μ m

RNA, protein, and secreted protein were upregulated in TSC-deficient neurons. CTGF is an extracellular matrix protein that as of yet is not a well-studied protein in the CNS. It has been noted to be upregulated in response to trauma, injury, and excitotoxicity in the CNS (Hertel et al., 2000; Schwab et al., 2001; Conrad et al., 2005). Although its function in brain development has not been well illuminated, CTGF expression has been used as a marker for subplate neurons in both humans and in rodents (Heuer et al., 2003). It has not yet been shown to be expressed in other CNS cell types in normal conditions, although it has been shown in stellate reactive astrocytes in response to focal injury (Schwab et al., 2005). Since subplate neurons line the grey-white matter border, secreted CTGF would be propitiously located to modulate oligodendrocyte development. Furthermore, CTGF expression is developmentally regulated, with its mRNA first detected at E16, progressively increasing through birth and reaching a steady adult levels by P7, at which time the levels are sustained (Heuer et al., 2003). To test whether CTGF is expressed in our mouse model, we immunostained the mutant and control sections for CTGF. Over the course of early postnatal development (P14 and P21), the number of cells expressing CTGF was significantly increased and qualitatively, each CTGF+ cell appeared to express higher levels of the protein in the mutants (Figure 3.9). These CTGF+ cells also displayed high mTOR activation, as shown by their high pS6 levels that were even higher than other neighboring *Tsc1*-deficient neurons (Figure 3.10; Meikle et al., 2007). These results indicate that CTGF levels were indeed upregulated in the white-grey matter junction region in the mutant mice.

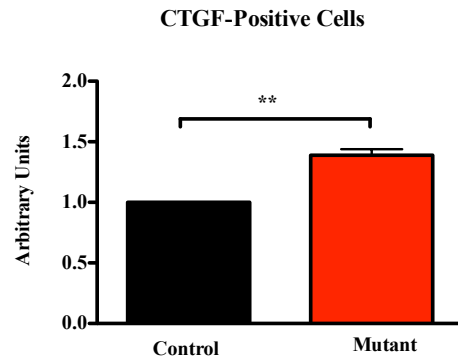
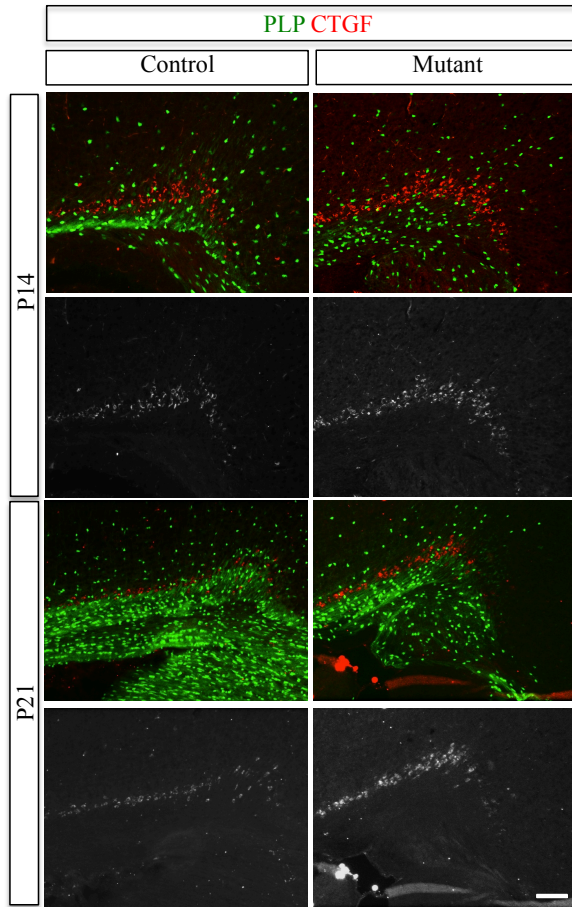


Figure 3.9. Immunostaining for CTGF in egfp-plp control and mutant brains shows CTGF is expressed along the grey-white matter border. Gray scale image shows the differences in number and intensity of CTGF expression. At P21, the CTGF levels remain high in the mutants. CTGF-positive cell numbers are increased by 1.4-fold in the mutants. Scale bar = 250 μ m; $p < 0.001$

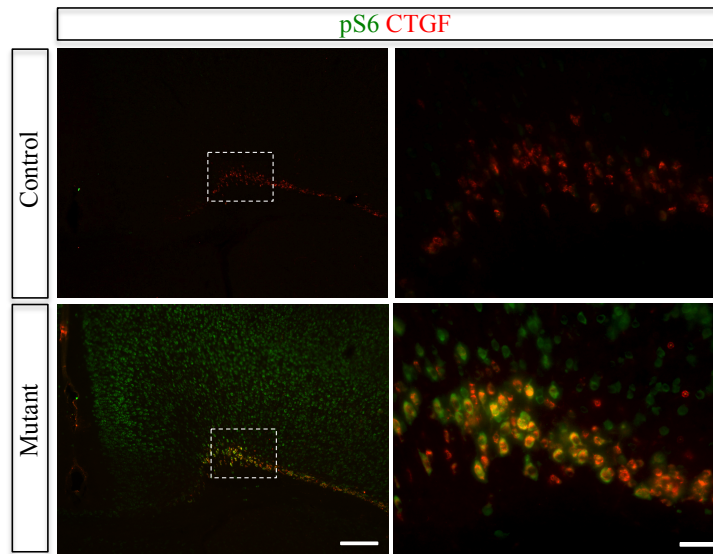


Figure 3.10. Mutant brains show co-localization of pS6 and CTGF along the grey-white matter border. Scale bar in left = 250 μm scale bar in right = 100 μm

In the single report on a role for CTGF in myelination, neuron-restricted *serum response factor (SRF)* deletion in mice inhibited oligodendrocyte differentiation. SRF is a transcription repressor of CTGF and deleting this in neurons, resulted in upregulation of CTGF and hypomyelination. Injection of adenovirus expressing CTGF reduced the number of MBP+ oligodendrocyte *in vivo* indicating that CTGF expression can be repressed by SRF (Stritt et al., 2009). To test the role of CTGF in our hands, we treated OPC cultures with purified recombinant CTGF protein and stained the cultures with MBP. We found a marked dose-dependent reduction in MBP-expressing oligodendrocytes when the cultures were treated with soluble CTGF protein, indicating that CTGF alone is sufficient to inhibit the differentiation of the oligodendrocytes (Figure 3.11).

As noted above, *Tsc2* deficient neuronal CM has elevated levels of CTGF. To investigate whether the CTGF in the *Tsc2*-deficient neuronal CM was necessary for oligodendrocyte maturation, we added 10 µg/ml anti-CTGF neutralizing antibody to the CM and immunoprecipitation to deplete the CM of CTGF. *Tsc2*-deficient neuronal CM, when depleted of CTGF, did not show an inhibitory effect on oligodendrocyte maturation, and the number of oligodendrocytes matured to MBP-positive cells significantly increased (Figure 3.12; TSH CM + AB = $76.77 \pm 3.5\%$; normalized to non-infected control). Anti-CTGF antibody had no effect on control CM (CT CM + AB = $84.87 \pm 7.6\%$). This data was further confirmed by myelin associated glycoprotein (MAG) staining that yielded similar results. Together these data suggest that neuronal CTGF is both necessary and sufficient to block oligodendrocyte maturation in culture. We next began to explore how secreted CTGF could inhibit

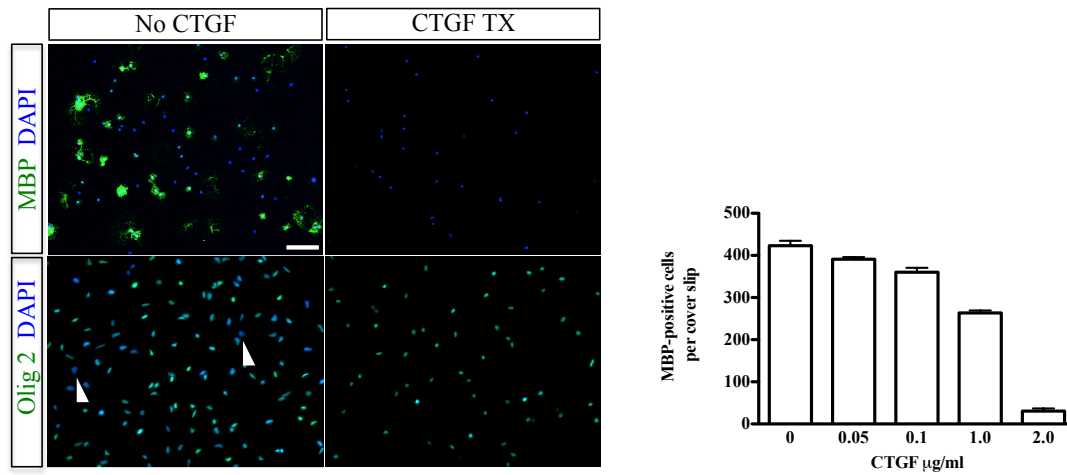


Figure 3.11. CTGF treated OPC cultures failed to express MBP in dosage dependent manner. Without CTGF addition, the OPCs spontaneously differentiated into MBP expressing oligodendrocytes in 4 days in vitro. CTGF treated cells that failed to differentiated all expressed olig2, showing they were lineage committed but remained at immature stage. In contrast, the control culture with MBP-positive cells had olig2-negative (arrowhead), indicating differentiation to mature oligodendrocytes. Scale bar = 100 μm

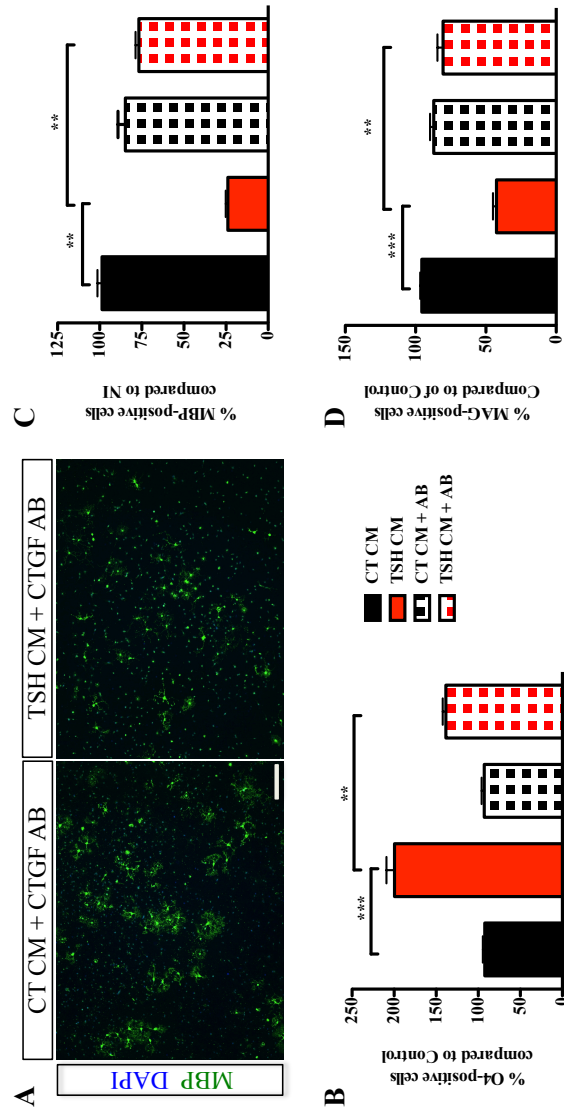


Figure 3.12. **A.** The OPC cultures were treated with CT CM, TSH CM, and CT CM, TSH CM with CTGF neutralizing antibody and after three days of treatment the cells were stained for O4, MBP, or MAG. **B.** The increase in O4+ cells in the TSH CM treated culture was decreased in the culture treated with addition of the neutralizing antibody (CT CM, 92.33 ± 2.4 ; TSH CM, 199.7 ± 9.7 ; CT CM + AB, 93.0 ± 3.1 ; TSH CM + AB, 138.7 ± 3.8). **C.** Neutralizing antibody addition increased the number of MBP-positive cells in the TSH CM treated culture (CT CM, 98.72 ± 2.7 ; TSH CM, 14.0 ± 1.2 ; CT CM + AB, 84.87 ± 8.4 ; TSH CM + AB, 76.7 ± 1.9). **D.** Similar effect was seen with MAG expression (CT CM, 95.7 ± 1.2 ; TSH CM, 42.5 ± 2.5 ; CT CM + AB, 87.6 ± 2.6 ; TSH CM, 80.6 ± 4.0). ** $p < 0.001$; *** $p < 0.0001$

oligodendrocyte maturation. CTGF belongs to the CCN family of matrix signaling modulators, which is named after three prototypical members, CCN1; CTGF, termed CCN2; and the nephroblastoma overexpressed protein (Nov), termed CCN3 (Perbal et al., 2004; Luft et al., 2008). The molecules are comprised of four modules: an insulin-like growth factor binding protein (IGFBP) domain (module I), a Von Willebrand factor domain (module II), a thrombospondin homology domain (module III), and a cysteine knot, heparin-binding domain (module IV). As an extracellular matrix molecule, CTGF is secreted into and sequestered in the matrix, available to interact with integrins on cells, growth factors, proteases, cytokines, and other extracellular matrix proteins, finely modulating their functions (Bornstein et al., 2002). Of the known binding partners of CTGF, IGF1 has been shown to promote oligodendrocyte differentiation, and CTGF binding would inhibit its ability to bind to oligodendrocytes, thus inhibiting its permissive role (Carson et al., 1993). IGF1 stimulates oligodendrocyte myelination in the CNS by activating the PI3K/mTOR pathway in the oligodendrocytes. If CTGF present in TSH neuronal CM prevents OPC differentiation by blocking IGF1 activity, then one would expect lower mTOR activation in CTGF-treated OPCs. To test this hypothesis, we stained WT OPCs treated with control versus TSH CM and found decreased number of pS6-positive OPCs in cultures treated with the TSH CM (Figure 3.13; CT CM, $101 \pm 5.9\%$; TSH CM, $64.7 \pm 4.6\%$; $p < 0.05$). Morphologically, *Tsc2*-KD CM treated-cells that were positive for pS6 usually appeared to be bi- or tri-polar, indicative of earlier stages of differentiation compared to the multiple processes and branching often seen in the

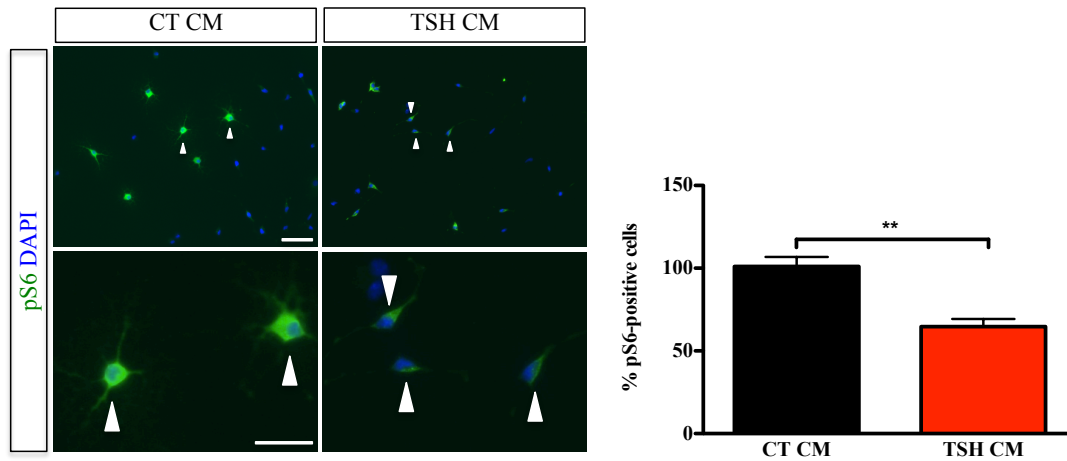


Figure 3.13. CT CM and TSH CM treated oligodendrocytes were stained for pS6, a marker for mTOR activity. Higher number of CT CM treated oligodendrocytes expressed pS6 compared to the TSH CM treated oligodendrocytes (CT CM, 101 ± 5.9 ; TSH CM, 64.7 ± 4.6 ; $p < 0.001$). CT CM treated oligodendrocyte appear morphologically further differentiated with multiprocesses compared to the TSH CM treated oligodendrocytes (arrowhead). Scale bar in top row = 100 μm, in bottom row = 50 μm)

control treated cells (Figure 3.13). These results are consistent with the hypothesis that TSH CM decelerates down the OPC differentiation, probably by IGF-1 inhibition. Since myelination could be a region specific process, we looked at the optic nerve to see if the myelination was compromised in parts of the CNS away from the CTGF-expressing cortex. Using the *Tsc1^{flox/flox}SynICre* mutant mice, we did not observe any significant differences in MBP levels of the optic nerves between the mutant and the control mice. We also did not observe any detectable levels of CTGF in the optic nerve of either experimental groups although it remains to be seen whether levels are upregulated in the retinal ganglion cells (Figure 3.14). We also did not see any compromise in Schwann cell myelination of the sciatic nerve (Figure 3.14). Given the long standing suspicion that oligodendrocytes may be regionally heterogeneous populations with varying differentiation properties, optic nerve and other white matter tracts that we did not investigate may show no deficits in differentiation regardless of the presence of CTGF.

Only one study has shown that CTGF regulation in neurons is in part through transcription factor SRF, which normally represses transcription of *ctgf* (Stritt et al., 2009). The most-studied pathway regulating CTGF expression is the TGF β signaling pathway via Smad and mitogen-activated protein (MAP) kinase (Burgess et al., 2005; Kwon et al., 2007; Fu et al., 2001; Massague and Wotton, 2000). TGF β stimulation leads to Ras/MAPK/ERKinase (MEK1/2) activation, which is required for CTGF promoter activation and measurable increase in secreted CTGF in fibroblasts (Leask et al., 2003; Leivonen et al., 2005). To test whether this is the mechanism of expression in neurons, we treated *Tsc2*-KD and control neurons with

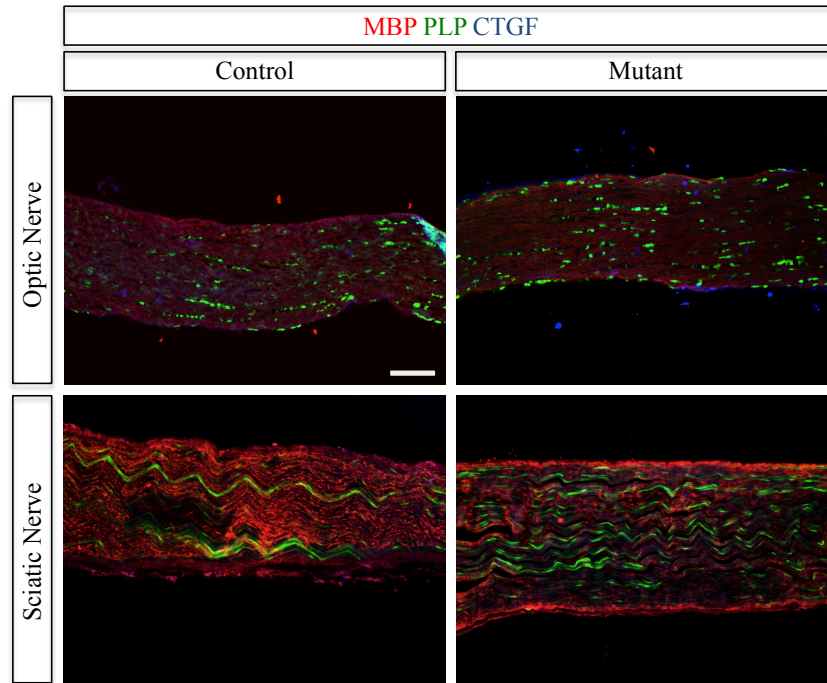


Figure 3.14. The optic and sciatic nerve from P21 eGFP-Plp+ control and mutant mice were stained for MBP and CTGF and showed no differences in myelination, oligodendrocyte numbers and CTGF expression. CTGF levels were almost undetectable in both nerves. Scale bar = 100 μ m

CI1040 and U0126, inhibitors of MEK1/2. We saw moderate but statistically insignificant decrease in CTGF (Figure 3.16), indicating that mTOR mediated upregulation of CTGF subjugates the effect of MEK1/2 regulation. We also asked whether PI3K, upstream of TSC/mTOR that has been shown to be important in CTGF expression is important in its neuronal regulation (Khoo et al., 2006). Additionally, PI3K activates Akt, and therefore is a modulator of many other signaling pathways in addition to mTOR such as GSK3 β and FOXO1. LY294002, inhibitor of PI3K had no effect on CTGF expression (Figure 3.16), indicating that other downstream effectors of PI3K/Akt do not regulate CTGF levels in neurons. In contrast, direct inhibition of mTOR by rapamycin treatment was able to reduce the CTGF protein levels dramatically, further suggesting that the CTGF increase is mTOR dependent in TSC background.

These findings point to CTGF as a potential therapeutic target. As a first step in investigating its potential, we wanted to confirm that it is expressed in humans. We observed CTGF expression from a resected tuber and from perituber tissues from an one-year-old patient with TSC. All CTGF positive cells were also pS6 positive although not all pS6-positive cells expressed CTGF, indicating that a subtype of TSC-deficient cells upregulate CTGF expression (Figure 3.15). Furthermore, we found CTGF to be upregulated in pS6-positive cells of a patient with periventricular leukomalacia (PVL) raising the possibility that the modulatory and anti-myelinating function of CTGF may extend beyond TSC patients.

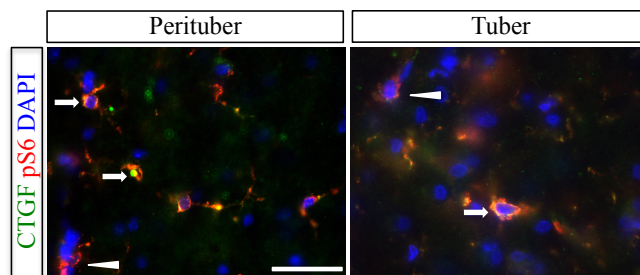


Figure 3.15. Tuber and perituber brain sections resected from 1 year old TSC patient were stained with CTGF and pS6. All CTGF-positive cells were also pS6-positive (arrowhead) showing mTOR regulation. Not all pS6-expressing cells were CTGF-positive (arrow). Scale bar = 100 μ m

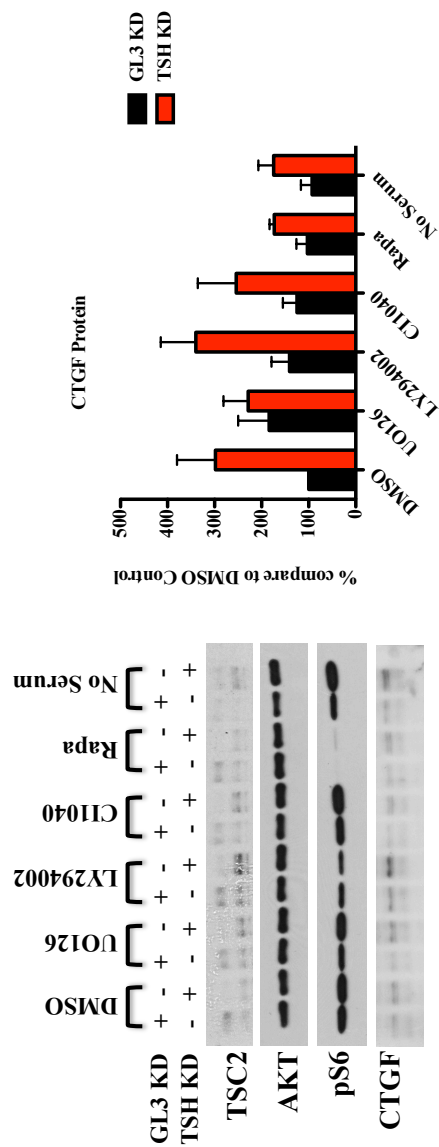


Figure 3.16. Rat cortical neurons were treated with GL3 or TSC2 (TSH) knockdown lentivirus, and treated with either DMSO, LY294002, CI1040, Rapamycin or B27 serum withdrawal overnight. As expected Rapamycin and serum withdrawal resulted in dramatic decrease in mTOR activity as shown in pS6 levels. This decrease corresponded to decrease in CTGF levels in the TSH KD neurons. LY294002 (PI3K inhibitor) had similar but more moderate effect, while LY294002 (PI3K inhibitor) and CI1040 (MEK1/2 inhibitor) seeming had no effect on CTGF levels. The samples were normalized to respective AKT levels.

Discussion

Hypomyelination in TSC patients has only recently garnered attention for possible role in cognitive deficits of the affected patients. Although various studies have begun to address the role of mTOR and its upstream/downstream molecules in oligodendrocytes development myelination, we are the first to present the evidence of neuronal-glial interaction in a TSC model. Our study shows that Tsc-deficient neurons upregulate CTGF along the grey-white matter border and that this factor is sufficient to arrest the differentiation of the OPCs *in vitro*. Conditioned media collected from Tsc-deficient neurons was able to replicate the arrest in differentiation *in vitro* and this was reversible using CTGF neutralization antibody, revealing CTGF as a novel therapeutic target in TSC and possibly other related diseases.

Inhibitory signals in myelination

There may be other contributing factors to hypomyelination in the neuronal deficient TSC model. mTORC1 is a master regulator of protein synthesis and its hyperactivation results in excessive translation of many mRNA transcripts (Ruvinsky et al., 2005). Our microarray analysis methods were stringent and thus eliminated many transcripts that were only slightly upregulated in the *Tsc2*-deficient samples, but even this slight change may be enough to have a physiological effect. An example of possible elevated inhibitory signal is the Wnt and bone morphogenic protein (BMP) pathway. Wnt/b-catenin and BMP pathways have been shown to inhibit oligodendrocytes differentiation (Shimizu et al., 2005; Feigensohn et

al., 2011). This is interesting in that Wnt and BMP are both binding partners of CTGF; it is concernable that CTGF may sequester Wnt and BMP and release them simultaneously in response to an unknown signal, creating a prohibitive environment for the maturation of oligodendrocytes. However, Wnt pathway has been shown to inhibit OPC generation in the telencephalon, and we saw no deficits in OPC numbers in our mouse model, rendering this pathway unlikely in our model (Langseth et al., 2010).

Another possibility is that the inhibitory mechanism is not a direct consequence of a signal regulating oligodendrocyte development but rather a premature demyelinating signal emanating from TSC-deficient diseased axons. However, our data demonstrating developmental arrest in oligodendrocyte lineage argues against an exclusive demyelination in our model; regardless, it remains to be seen whether TSC-deficient gliosis or axon degeneration generates signals that contribute to decreased white matter in the patients.

CTGF

CTGF is an extracellular matrix protein that belongs to CCN family of proteins that influence physiological mechanisms including adhesion, migration, differentiation, mitogenesis, and survival. Members of the CCN protein family are multimodular, with N-terminal secretory signal, IGFBP module, von Willebrand factor type C repeat, thrombospondin type I repeat (TSP) and a carboxy-terminal domain (CT) containing a cysteine knot. There are several upstream regulators of CTGF including TGF β , VEGF, and serum, all of which have respective modulatory

elements upstream of the CTGF promoter region (Grotendorst et al., 1997; Philippart et al., 2004; Sun et al., 2006; Muehlich et al., 2007). In this way, CTGF is an effector of other growth factors, fine-tuning their functions in a context-dependent manner, making generalization of its function difficult.

Pathologically, CTGF has been shown to be involved in both acute and chronic mechanisms including inflammatory responses, wound healing, and cancer (Hynes et al., 2009; Bornstein et al., 2002; Llera et al., 2010; Chiodoni et al., 2010). The long list of functions of this protein is specified by the location of secretion as well as the posttranslational modification that will alter its binding partner specificity. CTGF expression is most prominent and essential in the skeletal and cardiovascular systems of the developing mouse embryos, gain/loss of function studies showing that CTGF is necessary for lung formation, cartilage, and vascularization of the growth plate (Friedrichsen et al., 2003; Surveyor 1999; Fukunaga et al., 2003; Bonniaud et al., 2003 and 2004). In fact, CTGF-null mice are dead at birth presumably due to malformation of these critical physiological systems necessary for survival (Ivkovic et al., 2003). It is these dramatic physiological abnormalities in CTGF null mice that have thwarted interest in the protein's function in the central nervous system. My work begins to offer new insights and possible function of the protein in the CNS, and shows that full exploration of its regulation and role in the CNS may yield interesting information of a novel pathway.

Regulation of CTGF by mTOR in neurons

According to our results, CTGF is both transcriptionally and translationally upregulated in Tsc-deficient neurons. This leads to higher levels of secreted CTGF in the media of cultured cells but it is not yet clear whether the increased translated and secreted protein are direct reflections of upregulated transcription, or the latter two processing mechanism are separately increased by Tsc-deficiency as well. It is also possible that degradation of the protein is affected. Given the variety of processing, cell/region specificity, promiscuous binding properties of the protein and the unique characteristics of neurons, the known CTGF properties in other cell types may not apply in the CNS.

The promoter region of *ctgf* contains many transcription factor binding sites, including sites for SMAD, basal control element (BCE) 1, specificity protein 1 (Sp1), Ets-1 and hypoxia inducible factor (Hif) 1a (Leask et al., 2009). TSC1/2 regulates Hif1a, with loss of TSC2 function upregulating Hif1a activity (Brugarolas et al., 2003; Duvel et al., 2010). Therefore, it is possible that Hif1a increases the transcription of CTGF in TSC-deficient neurons. Whether these binding sites are activated in neurons in disease (e.g. TSC) or normal developmental mechanisms should be investigated. SRF is a known transcription repressor of *Ctgf* in neurons, able to selectively repress a number of genes even without external stimuli (Stritt et al., 2009). In our microarray experiment, we saw no indication of transcriptional suppression of other SRF target genes such as *c-fos*, *Cyr61*, *Acta1*, *Egr1*, suggesting that increased *Ctgf* in *Tsc2*-KO neurons is most likely not due to SRF inactivation. Additionally, when we withdrew serum from the neuronal media we observed surprising

decrease in CTGF transcripts in Tsc-deficient neurons as well as protein (data not shown; Figure 3.16), suggesting that the nutrient sensitive mTOR signal can override SRF. We additionally explored the TGF- β /MEK1/2 modulation of CTGF using inhibitors of MEK1/2 given that in fibroblasts, MEK1/2 activation is required for *ctgf* promoter stimulation (Leask et al., 2003). In fibroblasts, MEK1/2 activation and subsequent Smad3 and ERK1/2 phosphorylation lead to *Ctgf* transcription activation (Leivonen et al., 2005). MEK1/2 phosphorylates and thereby activates MAPK (also known as extracellular signaling-regulated kinase ERK); interestingly, ERK can also phosphorylate and inhibit *Tsc2* (Ma et al., 2007). Therefore, MEK1/2 inhibition should decrease CTGF levels, either through Smad3 or ERK1/2-TSC pathway. However, we saw only moderate decrease in CTGF in Tsc-deficient neurons indicating that mTOR is a MEK1/2-independent regulator of CTGF at least when it is hyperactive. In addition, we saw no decrease in control neurons with MEK1/2 inhibitors, indicating that there may be a novel mechanism of CTGF in the neurons.

Furthermore, while performing western and immunoprecipitation (IP) experiments in the current work we observed that cell lysates relatively contained small amount of CTGF compared to the conditioned media, indicating active secretion of the protein into the media. CTGF can either be secreted into the media or be associated with the ECM on the cell surface, and our current data shows that in neurons, CTGF appears to be mostly secreted (Chen et al., 2001; Kubota et al., 2001). Although we detected full length CTGF in the western blot, we also saw smaller proteolytic fragments in the IP samples when blotted (data not shown). Since the

full length CTGF contains four binding domains, post-translational modification that obliterates any of the domains would change the binding partner of the protein, and hence its function. Given our microarray evidence that *ctgf* transcripts are enriched in the axon, our previous work that show that the TSC/mTOR pathway regulates the synthesis of a subset of proteins in neurites, and the current evidence that CTGF can modulate the myelination of the axons, investigation into the secretory and local translation mechanisms would yield further insight into CTGF's neuronal regulation (Nie et al., 2010). The exact mechanism of CTGF regulation in the central nervous system is yet to be investigated.

The current work begins to uncover the basis of alterations in myelination that occur as direct result of TSC loss, and provide future directions in developing better-targeted therapeutic treatments for the numerous debilitating hypomyelinating diseases. This work takes a novel and innovative approach in that we began to look at how TSC-deficient cells can affect the development of a “normal” cell. How axons regulate OL differentiation and myelination in the CNS is a long-standing unanswered question, especially in disease models, and our work illuminates the therapeutic potential of studying non-intrinsic mechanisms of abnormal white matter development.

Chapter 4

The Role of Oligodendrocyte TSC/mTOR

Summary

TSC patients have systemic loss of TSC1 or TSC2 including in oligodendrocytes. Past studies in PTEN and AKT have shown that mTOR activation is critical for oligodendrocyte differentiation and myelin production. Hyperactivation of mTOR results in hypermyelination, but it is not yet known how TSC loss and subsequent hyperactivation of mTOR results in hypoactivation. To begin to explore this question, I have developed a culture system to efficiently knockdown *Tsc2* in oligodendrocytes and found that transcript levels of myelin proteins are downregulated in *Tsc2*-deficient oligodendrocytes, suggesting transcription regulation of mTOR in TSC background.

Introduction

TSC patients have loss of *TSC1* or *TSC2* not only in neurons, but in all cells. To obtain a more complete picture of the role of TSC1/2 in oligodendrocytes development and myelination, I have decided to complement my first project by studying the cell-autonomous roles of Tsc-deficiency in OLs. Tsc1/2 loss in

oligodendrocytes has not yet been studied despite observations of hypomyelination in both humans and mice. Intracellular mechanisms in oligodendrocyte alter its responsiveness to the extracellular signals and therefore are important regulators of their survival, maturation, differentiation, and ultimately their ability to myelinate. Recent studies have indicated that certain components of the PI3K-Akt-mTOR pathway are important for OL differentiation and myelin production. Over-expression of constitutively active Akt in OLs (*Plp*-Akt-DD mice) results in hypermyelination, which can be blocked by rapamycin treatment (Flores et al., 2008; Narayanan et al., 2009). In the same study, treatment of wildtype mice for 3 weeks starting at P21 also reduced myelination, indicating mTOR activity is necessary for myelination during this period of development (Narayanan et al., 2009). Second, conditionally deleting *Pten* in OLs (either *Pten^{ff}Olig2-Cre*; or *Pten^{ff}Cnp1-Cre*) results in hypermyelination despite the lack of relative increase in OL numbers (Harrington et al., 2010; Goebbels et al., 2010). Moreover, *Pten^{ff}Olig2-Cre* mice exhibited progressive dysmyelination and axonal degeneration by yet unidentified mechanism. All three of these studies focused on molecules upstream of TSC1/2. PTEN inactivation and Akt-DD overexpression would be expected to inhibit TSC1/2 function via phosphorylation of TSC2, but it is also important to remember that these upstream molecules have many other downstream pathways in addition to TSC, especially given its relevance to human disease.

Tsc1/2 loss in oligodendrocytes has not yet been studied despite observations of hypomyelination in both humans and mice. One of the causes of hypomyelination could be premature oligodendrocyte death, implicated in a

number diseases presenting with dysmyelination. Cells have evolved a number of defenses against oxidative damages incurred by ROS activities, including superoxide dismutases (SODs), peroxidases and catalases, and antioxidants. Interestingly, oligodendrocytes exhibit selective vulnerabilities to stressful stimuli dependent upon the stage of development, causing differential responses to pathologies that affect embryonic, perinatal, and late postnatal oligodendrocytes. OPCs are significantly more vulnerable to oxidative insults most likely due to their four-fold and eight-fold decrease in mitochondrial SOD and antioxidant capacities compared to mature oligodendrocytes, respectively (Baud et al., 2004). Overexpression of mitochondrial SOD, manganese SOD, in OPCs had protective effect against cell death in mild oxidative insult while more mature oligodendrocytes were more resistant to injury at basal levels (Baud et al., 2004). Similarly, glutathione peroxidase, which utilizes glutathione to detoxify peroxides, and active catalase levels were significantly lower in OPCs compared to oligodendrocytes, resulting in more caspase-3 activation and poly ADP ribose polymerase cleavage after mild oxidative injury (Khorchid et al., 2002). Interestingly, TSC1/2 loss results in mTOR-dependent ER stress response at baseline. Upon treatment with stress-inducing agents such as Thapsigargin, the Tsc2-deficient cells exhibited lowered threshold for induction of unfolded protein response (UPR) regulated genes and mitochondrial cell death pathways. More importantly, the lack of Tsc activity led to increased expression of pro-apoptotic transcription factor CHOP (C/EBP homologous protein), production of reactive oxygen species (ROS), and susceptibility to apoptosis. Together, it may be possible that Tsc-deficient oligodendrocytes undergo cellular stress response or

apoptose prematurely. Additionally, OPCs also have significantly lower levels of anti-apoptotic protein, Bcl compared to their mature counterparts (Khorchid et al., 2002). Taken together, these observations yield at least partial explanation to how and when diffuse white matter diseases characterized by reduced white matter volume and abnormal myelination may develop. Selective deaths of OPCs during late embryonic period when these cells are actively proliferating would result in severe and lasting disruptions in myelination. Indeed, it has been shown that the time of peak incidence of periventricular white matter injury coincides with the gestational time when OPCs are the predominant cell type in humans (Back et al., 2001).

We began to explore these numerous possibilities by reducing the expression of TSC1/2 protein complex in cultured oligodendrocytes. We found that using the Tsc2 knockdown lenti virus, we were able to ablate 90% of Tsc2 transcripts within five days of infection, and that this ablation leads to increase in ER stress markers. Interestingly, the transcripts of myelin related proteins were decreased. These results show the possibility that the mechanism of hypomyelination in Tsc-KD oligodendrocytes may be different from PTEN-cKO or Akt-DD models, and needs to be investigated more carefully in the future.

Results

We first wanted to test the efficiency of the Tsc2 KD virus on the oligodendrocytes. We found that the oligodendrocytes were more vulnerable to the virus and the infection period had to be shortened from 6 hours to 4 hours to ensure

cell survival. After five days of infection, Tsc2 mRNA is decreased by about 80% compared to control infected oligodendrocytes, and the cells continued to be healthy for at least another week thereafter.

mTOR activation by either PTEN inactivation or Akt activation has been shown to increase myelination by increased transcription and translation of the myelin related proteins (Harrington et al., 2009; Narayanan et al., 2009). Since TSC inhibits mTOR activity, we predicted that transcription of the myelin related genes would be upregulated in Tsc-deficient oligodendrocytes. To our surprise, we saw a significant decrease in mature myelin genes *cnp*, *mbp*, and *plp* (Figure 4.1). Given our previous finding that Tsc-deficient cells are susceptible ER stress, we tested for heme oxygenase 1 (HO-1) transcript levels that should be upregulated in response to cellular stress. Indeed, we saw significant increase in HO-1 transcript levels in Tsc-KD oligodendrocytes. Together these findings show that Tsc deficiency causes downregulation of mature oligodendrocyte genes and upregulation of a cellular stress marker.

These findings confirmed the need to take a broad approach to identify the downstream effectors of TSC that have not been considered in prior research into mTORC1/2 effectors. Therefore we took a genome wide approach and performed microarray analysis on Tsc2-deficient oligodendrocytes versus control oligodendrocytes with or without rapamycin (microarray not shown). None of the top genes that are differentially regulated in our array were obvious oligodendrocyte development regulators, paving the way for future studies to look more closely into unpredicted genes that regulate white matter development.

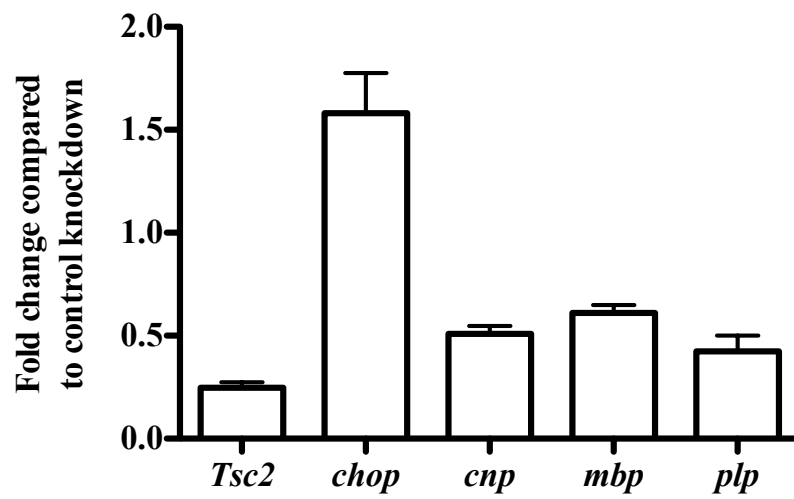


Figure 4.1. mRNA expression levels in oligodendrocyte after five days of lentivirus *Tsc2* knockdown. Increase in ER stress marker *chop* accompanies ~80% decrease in *Tsc2* and significant decreases in myelin protein genes.

Discussion

We began to investigate the role of TSC/mTOR pathway in oligodendrocyte development and myelination. Our preliminary findings provide surprising results considering existing literature and solidify the need to investigate in depth the genes that change with loss of TSC function that have not yet been considered.

Since TSC loss results in hyperactive mTORC1, the obvious hypothesis would be that TSC-deficient oligodendrocytes would hypermyelinate *in vivo* similar to the Akt-DD and Pten mutant mice that upregulate mTORC1 in oligodendrocyte. However, the transcriptional repression of Mbp and Plp in TSC-deficient oligodendrocytes shows that sustained loss of TSC may result in repression of mTORC2 activity. While TSC1/2 negatively regulates mTORC1, it promotes mTORC2 activity in a Rheb-independent manner that might involve the direct binding of the TSC1/2 complex to components of the mTORC2 complex (Alexander et al., 2010; Sahin et al., 2010; Khwaja et al., 2011). mTORC1 and mTORC2 can both increase protein levels but only mTORC2 can modulate the transcription of *mbp* and *plp* (Tyler et al., 2009). Our results show that prolonged attenuation of TSC activity results in suppression of *mbp* and *plp*, suggesting the possibility that contrary to the expected, the TSC-deficient oligodendrocytes may hypomyelinate *in vivo*. Our lab is currently generating the *Tsc1^{f/f};Plp-Cre* mouse to test this hypothesis.

Considering the OPCs are selectively vulnerable to excitotoxicity, we hypothesized that the TSC1/2-deficient OPCs may undergo premature apoptosis. Although we have not yet investigated whether the OPCs are dying, increase in HO-1 suggests that the OPCs are undergoing cellular stress. Insulin-like growth factor I

promotes oligodendrocyte progenitor survival and it has been shown that the downstream pathway is PI3K-dependent and one of its critical downstream effectors is Akt, an upstream modulator of TSC2 (Cui et al., 2005). Inhibition of PI3K pathway results in increased cleaved caspase-3, reflecting activation of apoptotic pathway similar to our recently demonstrated data with Tsc deficient neurons (Di Nardo et al., 2009). If there is increased apoptosis, it remains to be seen whether the same mechanism is in place.

In addition to cell death, proliferation/differentiation/myelination are also susceptible to perturbation and they can be modulated by mTOR activity. mTOR activation is required for the terminal differentiation of the oligodendrocyte precursor cells (OPCs) by enabling intrinsic mechanisms to acquire OL-specific gene expression (Inoki et al., 2002). Interestingly, mTORC1 and mTORC2 appear to have distinct temporal roles in the process; mTORC1 targets P70S6K1 and 4E-BP for phosphorylation at the onset of OPC differentiation while mTORC2 substrate Akt Ser473 phosphorylation was sustained through latter stages of differentiation (Inoki et al., 2002). Additionally, other recent studies have shown that hyperactivation of the pathway in OL lineage cells either by selective deletion of PTEN (*Olig2-cre, Pten^{fl/fl}*) or constitutive overexpression of Akt (*PLP-Akt-DD*) both result in mTOR-dependent hypermyelination (Nellist et al., 2001; Leone et al., 2003). PI3K/mTOR pathway appears to regulate myelination by modulating the amount of protein translation; however, this process seems to be limited to the developmental period, as *Olig2-cre, Pten^{fl/fl}* mice did not exhibit greater remyelination following lysolecithin induced demyelination (Nellist et al., 2001).

This brings into light whether TSC/mTOR pathway can be applied to the recapitulation theory in the case of myelination. Although axons require revival of the mTOR pathway for successful regeneration, the remyelination process seems to be more complicated (Park et al., 2008). Therefore, alternative genes and proteins that modulate myelination may illuminate new candidates that regulate remyelination.

Chapter 5

TSC Localization to Peroxisomes in Neurons

Summary

Majority of the mutations in TSC proteins reside in their peroxisome targeting signals (PTS). Since intracellular trafficking of mTORC1 has recently emerged as an important factor in its proper function, we asked whether TSC localization to a subcellular organelle, peroxisome is also important in neurons. We found that mutation in PTS results in abrogation of function of TSC, and in neurons this resulted in loss of neuronal polarity, similar to the phenotype seen in TSC-deficient models. These results show that localization of TSC to the peroxisome is important for its proper function.

Introduction

My work has also extended into other neurological disorders that are attributed to TSC1/2 dysfunction. In collaboration with the Dr. Cheryl Lyn Walker's lab, we explored the relationship between TSC and peroxisome function. TSC1/2 has been shown to repress mTORC1 activity in response to oxidative stress via ATM (Ataxia Telangiectasia mutated; Alexander et al., 2010). Activation of ATM by reactive oxygen species (ROS) results in LKB1 phosphorylation, which in turn phosphorylates AMPK to activate TSC2 and repress mTORC1 (Alexander et al., 2010). Recently, intracellular localization and trafficking of mTORC1 has emerged as an important determinant of its activity. When mTORC1 is stimulated by amino acids, Rag GTPases of the Ragulator complex that usually localizes mTORC1 to the late endosome/lysosome compartments brings this kinase in proximity to the mTORC1 activator, Rheb (Korolchuk et al., 2011; Sancak et al., 2008; Sancak et al., 2010). This subcellular localization is crucial in proper activation of mTORC1, but whether there are other compartments where TSC1/2 localizes and what their function is at those sites have not yet been clear. Non-sense mutations of TSC1 and TSC2 result in complete inactivation of the mutated allele, but approximately 25% of TSC2 and 1% of TSC1 are non-terminating missense changes (Sancak et al., 2005). Of these, mutations in the PTS1 are some of the most common and here we show that this mutation dramatically reduces the localization of the TSC to the peroxisome (Coevoets et al., 2009).

Peroxisomes are subcellular organelles, similar to mitochondria in their ability to autonomously replicate. Peroxisomes carry out critical metabolic functions

such as β -oxidation of fatty acids, prostaglandins, and leukotrienes, as well as biosynthesis of bile acids (Wanders and Waterham, 2006). Peroxisome Biogenesis Disorders (PBD) are autosomal recessive diseases caused by genetic defects in key proteins that are part of the peroxisome function (Wanders and Waterham 2006; Weller et al., 2003). In the central nervous system, PBD often present as leukodystrophies, which are inherited conditions involving damage to the white matter of the brain. Of the disorders, Zellweger Syndrome is the most severe. The genetic mutations in peroxisome (PEX) genes results in absence of peroxisomes and/or defects in their organelle assembly, which result in hypotonia, liver disease, renal cysts and early death by age of one (Brosius and Gartner 2002). The peroxisome loss also results in severe neurological symptoms such as neonatal seizures, hypotonia, and brain dysgenesis, but the mechanisms underlying these symptoms have been unclear. In the CNS, the Zellweger syndrome is also associated with aberrant neuronal migration, positioning and hypomyelination (Steinberg et al., 2006).

Our collaborators have found that TSC1 and TSC2 proteins both contain peroxisome targeting signals (PTS), recognized by peroxisomal import receptors that traffic the many peroxisomal proteins and enzymes. Mutations in the PTS sequences abrogate delivery of TSC to the peroxisome, disabling ROS-induced mTORC1 repression. My results show that this defect resulted in abnormal neuronal morphology, providing a link between localization of TSC to the peroxisome and proper neuron development.

Results

A hallmark of Zellweger neuropathology is the presence of abnormal neuronal morphology and migration resulting in areas of polymicrogyria (Steinberg et al., 2006). How the loss of peroxisomes causes these phenotypes remains elusive, but it has been speculated that accumulation of very long chain fatty acids (VLCFA) and branched chain fatty acids (BCFA) that are usually degraded by peroxisomes could alter normal function of other organelles and cellular signaling, resulting in cortical dysgenesis (Ho et al., 1995). To test whether TSC1/2 co-localized at the peroxisome, we expressed flag-tagged full-length TSC1 and TSC2 in hippocampal rat neurons. Co-staining with antibodies to the FLAG tag and peroxisomal markers PMP70 and catalase showed localization of the TSC1/2 heterodimer components to the peroxisome in the neurons, consistent with our collaborators' findings in HeLa and HEK293 cells (Figure 5.1). These results indicate that TSC1 and TSC2 proteins localize at least partially to the peroxisome component in neurons.

Our lab in the past had found that TSC1/2-deficiency leads to multiple axon phenotype by increased mTORC1 activity and subsequent SAD-A kinase protein translation (Choi et al., 2008). Therefore, we wanted to test whether TSC2 expression that lacks functional PTS sequence and hence the ability to localize to the peroxisome may result in aberrant morphology as well. To investigate this, we performed functional assay of neuronal polarity in cells expressing FLAG-tagged WT-TSC2 or three different PTS1 mutations at a.a. 1743 (R1743Q, R1743G, R1743W), corresponding to three naturally occurring pathogenic mutations associated with TSC disease. Our collaborator had demonstrated that in non-

neuronal cells, these three pathogenic mutants fail to localize to the peroxisomes. Overexpression of wildtype TSC2 plasmid resulted in an increase in neurons without axons compared to controls expressing GFP alone, as previously shown (Figure 5.2; Choi et al., 2008). In contrast, expression of TSC2 lacking a functional PTS1 (TSC2 RQ, RW, and RG mutants) significantly reduced the fraction of cells with no axons and increased the number of cells with multiple axons. This finding provides a link between loss of peroxisomes, aberrant TSC signaling and neuronal pathophysiology of PBDs.

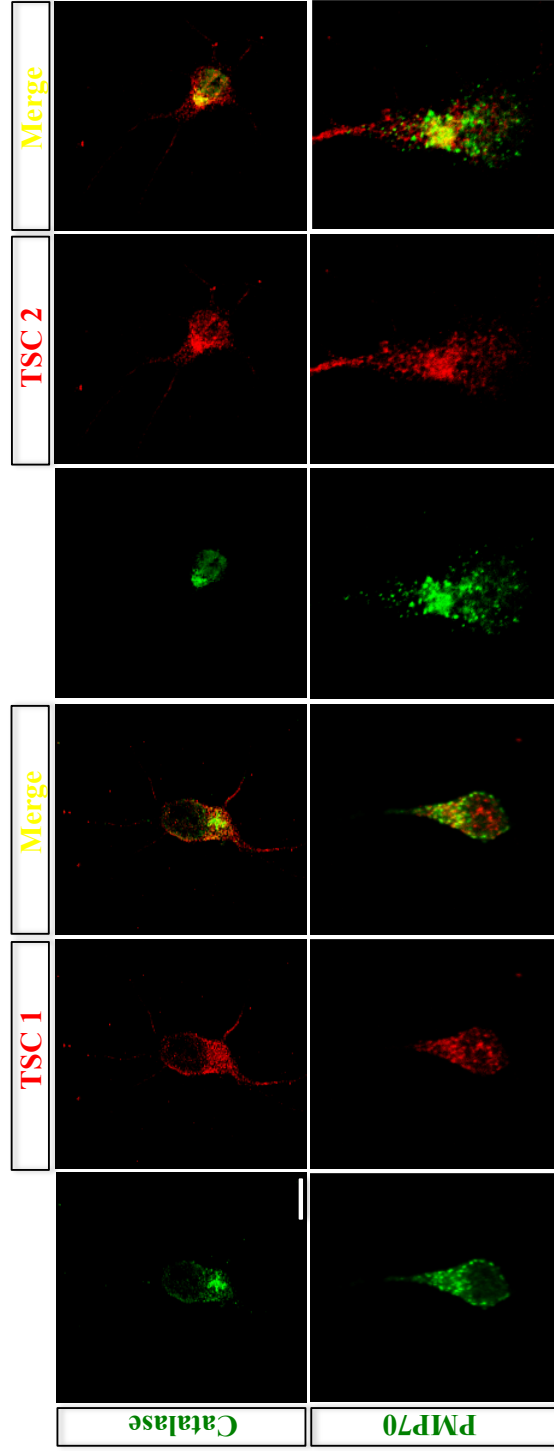


Figure 5.1. Rat hippocampal neurons were infected with flag-tagged full length TSC1 and TSC2, and stained for PMP70 and catalase, peroxisomal markers. TSC1 and 2 co-localize with the peroxisome proteins. Scale bar = 10 μ m

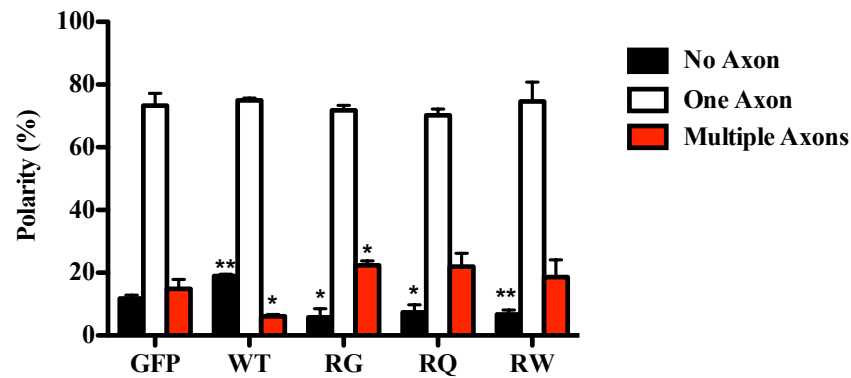


Figure 5.2. Rat hippocampal neurons were infected with GFP, full length flag-tagged TSC2, or TSC2 with mutations in the peroxisome targeting signal (RQ, RG, RW). 5 DIV, the neurons were stained with axonal marker Tau1 and categorized into no axon, one axon, multiple axon groups. PTS mutated TSC2 increased the multiple axon polarity and decreased the number of neurons with no axons.

Discussion

The current findings show that in neurons, TSC 1 and 2 are localized to the peroxisomes, and mutations that abrogate this localization results in loss of axon specification. Although the accumulation of VLCFA could alter cellular signaling, mice with peroxisomal beta-oxidation deficiency (Pex5 knockout) show no neuronal migration defects that exist in the Zellweger syndrome mice (Baes et al., 2002). Additionally, neuronal migration was not improved with supplementation with polyunsaturated fatty acid DHA, indicating that an additional signaling mechanism is necessary in the neurons (Faust et al., 2001). Our findings provide an explanation that TSC localization to peroxisome and subsequent regulation mTORC1 can affect neuronal morphology and development.

Our collaborators' work shows that inability for TSC to localize with the peroxisome results in failure to repress mTORC1 in response to ROS. Although this has not been shown in neurons, given the similarities across the cell types examined so far combined with our past finding that Tsc-deficient neurons have elevated levels of ER stress, dysregulated mTORC1 and its downstream effectors are likely in neurons as well. Together, these observations suggest an alternative explanation for the aberrant neural development in Zellweger patients including axon guidance, formation and myelination. While PBDs and TSC are quite divergent genetically, some of the cellular mechanisms of these very different diseases may converge on a common pathway that results in similar phenotypes. It remains yet to be seen whether the neuronal defects our lab has demonstrated in cultured neurons would be replicated in Zellweger mice or patients.

Chapter 6

Discussion

TSC is a high penetrance disease, causing fundamental alterations that persist throughout life. Much of the focus in the field has been on astrocytes and neurons, and it was the goal of my thesis to begin to uncover the mechanism underlying abnormal white matter development in the disease. In doing so, I took an innovative approach by studying the non cell-autonomous role of the TSC/mTOR pathway and discovered a novel paracrine signaling molecule that was dysregulated in TSC-deficient neurons (Figure 6.1). This body of work reveals possible new roles for subplate neuron development in TSC, a new molecular player in CNS-CTGF, and the importance of intracellular localization of TSC.

Origin of hypomyelination in TSC

The first goal of my thesis was to identify the cause of hypomyelination in our neuronal KO mouse model. From our previous work, it was clear that hypomyelination in TSC arises early in development, negating the possibility of demyelination (Meikle et al., 2007). I was able to show here that the brains of the neuronal mutant model of TSC exhibit a dramatic decrease in number of mature

oligodendrocytes due to their arrest in differentiation. Using an unbiased genome-wide approach, I identified a novel molecule, CTGF, which is dysregulated in Tsc-deficient neurons and is sufficient to cause the arrest in differentiation of the immature oligodendrocytes. From the human TSC patient tissues, we verified that CTGF is also overexpressed in mTOR hyperactivated cells, but this data is too premature to suggest that CTGF is relevant to the patients' hypomyelination or that it is expressed close to the regions of white matter deficits. However, the data together provides convincing evidence that extrinsic signals emanating from Tsc-deficient cells could be as detrimental as an intrinsic loss of TSC. Therefore, the aberrant paracrine signaling is an important and necessary concept that has not at all been explored in TSC or in other developmental disease models, such as Zellweger disease, that was investigated in chapter 5. Although these two diseases arise from very different mutations, they are similar in that they both result in dysfunction of TSC protein. In Zellweger syndrome, TSC is unable to localize to the peroxisome and this results in an inability to properly form a polarized neuron. Furthermore, our collaborators have found that without its ability to localize to the peroxisome, the capacity of TSC to inhibit mTOR is also diminished. Because Zellweger syndrome also presents with hypomyelination, further understanding of neuronal-TSC dysfunction could have more broad implications. A great starting point may be to take another unbiased approach by performing mass spectrometry using the conditioned media from the control versus TSC knockdown neurons to investigate which secreted signals are highly differentially regulated between the two groups. The target candidate proteins would have to be individually explored

for their functional role in disease manifestation, but such unmitigated approach would provide a valuable starting point for future research.

Systematic loss of TSC via mutation results in heterozygosity in patients. Somatic independent mutation, second hit, and thus loss of heterozygosity is attributed to the most severe characteristics of the disease, such as tumor development, subependymal giant cell astrocytomas, and renal angiomyolipomas (Carbonara et al., 1996; Henske et al., 1997; Chan et al., 2004). Interestingly, loss of heterozygosity is rarely seen in cortical tubers, which suggests that haploinsufficiency and the subtler biochemical aberrance it causes is enough to give rise to the CNS abnormalities (Chan et al., 2004). As we have seen in the *TSC2*^{+/-} model, haploinsufficiency is sufficient to result in miswiring of the retinogeniculate axons in otherwise healthy animals, accentuating the elaborate nature of CNS development and its vulnerability to even small molecular changes (Nie et al., 2010). Hence, it is conceivable that minor changes in extracellular signals could have a greater impact on haploinsufficient cells than on normal cells. In fact, focal white matter deficits are often seen in subcortical regions underlying tubers. If all oligodendrocytes are haploinsufficient, conglomeration of dysplastic neurons, giant cells, and misshapen astrocytes that constitute tubers may provide an abnormal extrinsic environment for oligodendrocyte development (Calcagnotto et al., 2005; Makki et al., 2007). This may explain why not all white matter tracts are equally affected. Of course it is also possible that some oligodendrocytes undergo somatic second hit mutation. Nonetheless, TSC patients with the same genetic mutation can present with a wide spectrum of clinical symptoms, prompting the

need to isolate the mechanism underlying the phenotypes, rather than using an one-size-fits-all approach. Of the phenotypes, myelination is an attractive therapeutic target considering that other neurological manifestations develop *in utero*, making treatment difficult (Levine et al., 2000). In contrast, myelination occurs predominantly in postnatal life, and recent studies have demonstrated a surprisingly high level of myelin plasticity in the adult CNS (Bengtsson et al., 2005; Scholz et al., 2009).

The most apparent discrepancy in the TSC/mTOR myelin studies is that hyperactivation of mTORC in oligodendrocytes results in hypermyelination but hyperactivation of mTORC in neurons results in hypomyelination. TSC patients have systematic mutation resulting in loss of *TSC1* and *TSC2* and subsequently hyperactivation of mTORC in all cells, prompting whether TSC/mTOR pathway in one cell can override another signal. Given the results of my work, it could be that since *Tsc1*-deficient neurons inhibit differentiation, the ability for *Tsc1*-deficient oligodendrocytes to hypermyelinate by excessive production of myelin may not come into play (Figure 6.1). It also could be that there are other signaling pathways the previous studies have not yet considered. The studies that show increased mTOR leading to hyperactivation focused on molecules upstream of TSC1/2, PTEN and AKT (Narayanan et al., 2009; Tyler et al., 2009; Flores et al., 2008). PTEN inactivation and Akt-DD overexpression would be expected to inhibit TSC1/2 function via phosphorylation of TSC2. However, it is important to remember that Akt has many other substrates in the cell. In fact, one of them, GSK3 β , can inhibit OPC differentiation into oligodendrocytes (Azim et al., 2011). Thus, Akt

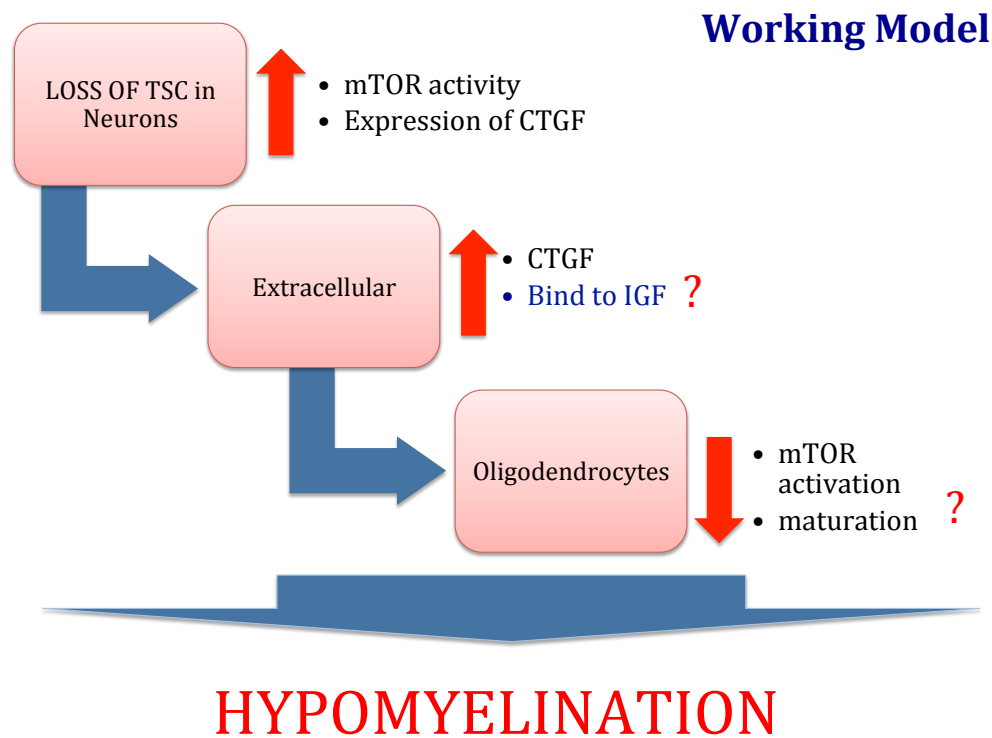


Figure 6.1. The working model established through our work. The current work shows that loss of TSC function in neurons results in upregulation of CTGF expression, which correlates to its increased levels in the extracellular matrix. Excessive CTGF can potentially bind to a number of factors, but its binding to IGF is most consistent with the decrease in myelination phenotype we observe in the TSC-deficient neuron's conditioned media treated oligodendrocytes. TSH CM treated oligodendrocytes show decreased differentiation as well as decreased mTOR activation, suggesting that reduced differentiation arises from decreased translation of the myelin related proteins. This model predicts that in TSC patients who are haploinsufficient, TSC loss in neurons precedes chronologically the TSC loss in oligodendrocytes, which may actually result in hypermyelination due excessive mTOR activity in the oligodendrocytes. Because oligodendrocytes may never reach the maturation level to express the myelin proteins, TSC-deficiency in oligodendrocytes may not be reflected in the humans.

hyperactivity may partially work through inhibition of GSK3 β , as well as TSC1/2. If that is the case, then *Tsc1* deletion in oligodendrocytes may not be sufficient to induce hypermyelination. Furthermore, *Tsc1* deletion in oligodendrocytes would increase mTORC1 activity similar to *Pten* deletion or Akt-DD overexpression, but it would have a very different effect on Akt signaling. Most notably, TSC/mTORC-dependent negative feedback mechanisms exist to dampen the activation of upstream components of the network. The best characterized of these is the mTORC1- and S6K1-mediated phosphorylation of the insulin receptor substrate (IRS-1), leading to a block in insulin and IGF1 signaling to PI3K (O'Reilly et al., 2006; Manning et al., 2005). Thus, in *Tsc1*-deficient OLs, Akt phosphorylation and activity should be reduced. Furthermore, as a consequence of these feedback mechanisms rapamycin treatment increases the activation of PI3K and, under most conditions, results in increased Akt phosphorylation and activation. Based on these observations, it is difficult to predict what the effect of *Tsc1* or *Tsc2* deficiency would be on OL differentiation and myelination. Successful development of oligodendrocytes-specific conditional knockout mouse of *Tsc* will provide valuable and necessary answers to these discrepancies.

An additional insight from these studies is that mTORC-hyperactivated oligodendrocytes exhibit progressive dysmyelination that eventually spreads to axonal degeneration (Harrington et al., 2010). It is not yet known whether the patients also suffer from dysmyelination, but given haploinsufficiency of the oligodendrocytes in the patients, this may be a contributing factor in their white matter abnormalities.

Effect of Rapamycin

One of the surprising findings of this study and of our previous work (Meikle et al., 2008) is the lack of effect that rapamycin treatment had on the control animals. At the time publication of our previous paper, it was not yet known whether mTOR directly regulated development and expression of myelination proteins in the oligodendrocytes. Since then, a number of papers have shown that inhibition or overactivation of components of the PTEN/Akt pathway that leads to mTOR hyperactivation, also results in hypermyelination (Flores et al., 2008; Tyler et al., 2009; Narayanan et al., 2009; Harrington et al., 2010). Although these findings were informative and necessary, they were not surprising in that mTOR is a known protein translation regulator. Therefore, an increase in its activity would be expected to directly correlate with increased myelin protein production. Conversely, inhibition of mTOR using rapamycin was found to inhibit translation of myelin proteins and cause hypomyelination in the wildtype brains (Narayanan et al., 2009). The authors of this study noted that our 2008 paper did not clearly show or note whether there was a myelin reduction in the wildtype mice with rapamycin treatment, and that they would expect it to decrease according to their findings (Narayanan et al., 2009; Meikle et al., 2008). My current work in chapter three shows that rapamycin treatment does not cause a reduction in myelin levels in our control mice, which brings into light the discrepancy between my findings and theirs, but there are key disparities in the methods that may explain these differences.

In the Narayanan paper, the rapamycin treatment was at 10mg/kg, 5 days a week, for 3 to 6 weeks depending on the experiment (Narayanan et al., 2009). This is a much higher dosage, longer and more frequent treatment protocol compared to the one used in our studies: 6mg/kg, every other day, for two weeks. Therefore, the reduction in myelination seen in their mice can be attributed to much stronger repression of mTOR that effectively hypoactivates all oligodendrocyte mTOR activity. Whereas the dose we used may have been just enough to assuage the hyperactivated levels of mTOR in the neurons without perturbing the intrinsic levels of mTOR in oligodendrocytes. Accordingly, they also observed overall reduction and tight packing of the neurons in rapamycin-treated wildtype brains that we could not detect in our wildtype brains, suggesting that at their dosage protocol, wildtype levels of mTOR activity are also strongly repressed in the neurons. This also illuminates the possibility that there exists a broad buffer zone of mTOR activity at which the cells can sustain homeostatic levels of protein translation activity. This phenomenon was also observed in my current work on axon size. The EM images in chapter three shows that the myelinated axons were very enlarged and the sizes were reversed by rapamycin treatment. However, there were many small (0.1-0.3 μm diameter) unmyelinated axons that did not increase in size in the mutants, and were resistant to rapamycin treatment in both mutants and the wildtype animals. This indicates that a subset of neurons, although deficient in TSC, did not become enlarged and therefore did not respond to rapamycin treatment. Narayanan et al. also noted an analogous observation in oligodendrocytes: although MOG, MAG, and CNP expressions were reduced with rapamycin treatment, they saw no significant

differences in MBP, PLP, and DM20 levels, which led them to conclude that perhaps the protein levels were already so high that they were unaffected by mTOR inhibition (Narayanan et al., 2009). An alternative explanation, however, is that because these proteins were differentially modulated when mTOR was repressed, there may be an intricate homeostatic mechanism that allows mTOR to select which proteins to continue translating when inhibited below its normal levels. Whether there is a rapamycin dosage effect on myelin protein levels would be an interesting future study. Furthermore, investigating how rapamycin treatment affects other glial types such as astrocytes would yield significant insights into cell-specific responses to rapamycin treatment.

Another key difference is the timing of the treatment. Narayanan et al., initially treated their mice at 12 weeks of age when the myelination process was completed and the oligodendrocytes were at a maintenance stage, and noted a slight reduction in MOG, MAG and CNP levels (Narayanan et al., 2009). They observed a much more robust reduction in CNP, MOG, PLP, DM20, and MBP expressions when they treated P21 mice for three weeks, and concluded that mTOR activity at a young age can modulate myelin amounts (Narayanan et al., 2009). Our mice were treated starting at P7 through P21 when the oligodendrocytes are more likely to be actively proliferating and differentiating; therefore, translation inhibition at this age may not be as critical to myelin protein amounts as we saw in our treated wildtype mice. Again, this could also be simply due to the lower dose of the drug we used. Regardless, rapamycin treatment prior to active myelin production seems to have a negligible effect on OPC proliferation, but inhibits differentiation into the mature

oligodendrocytes (Tyler et al., 2009). However, these studies were done *in vitro* only, and given that the hypomyelination in our model is a result of neuronal Tsc-deficiency, the effects of rapamycin are difficult to compare between the two studies. For instance, the authors noted that there are no changes in olig2 levels in response to rapamycin treatment, decoupling the relationship between olig2 and oligodendrocyte mTOR (Tyler et al., 2009). We saw increased olig2 in our mouse model and significantly increased expression of sonic hedge hog (SHH) in the Tsc-deficient neurons (data not shown), indicating the importance of neuron-secreted signals for oligodendrocyte maturation. Given the differences in the timing/dosage of rapamycin treatment, *in vitro/in vivo* model, and normal/abnormal neurons, the real effect of any mTOR inhibitor would have to be more closely investigated before being translated for therapeutic uses, especially given mTOR's ubiquitous expression and function.

CTGF in subplate neurons

Our work also implicates a new function for subplate neurons in white matter development. It also suggests the possibility that a dysfunctional subplate could contribute to erroneous circuit formation and pathfinding in TSC brains. Subplate neurons are some of the first-born cells of the cortex (Luskin and Shatz 1985). Once believed to be a mere transient population, subplate neurons are now believed to be critical players in developmental plasticity, maturation of cortical inhibition, and pathfinding of thalamocortical projections (Kanold 2009; Kanold and Shatz 2006; Ghosh et al., 1990; McConnell et al, 1989). Injury to subplate neurons

has been attributed to autism, attention deficit disorder, dyslexia, and schizophrenia, as well as hypoxic injuries that lead to periventricular leukomalacia (Volpe 2001; McQuillen et al., 2003). In rodents, there are at least six different subplate neuronal subtypes as characterized by morphological, electrophysiological, and neurochemical properties, and projection sites (Hanganu et al., 2002; Hoerder-Suabedissen et al., 2009; Friauf and Shatz 1991; Molyneaux et al., 2007). CTGF has been used as a marker for subplate neurons in rodents, although its function was not understood. The findings of our current study suggests that CTGF-positive subplate neurons could normally play an inhibitory role in limiting excess differentiation of oligodendrocytes during development. One possibility, given its time of expression, is that CTGF inhibits premature spontaneous differentiation of oligodendrocytes, allowing the oligodendrocyte precursor cells to develop appropriately and undergo their full number of intrinsic cycles of proliferation (Durand et al., 2000). It could sequester secreted IGF1 until the precursors have proliferated fully, then release it to aid in the differentiation. Of course, it is possible that CTGF is only expressed at inhibitory levels in the diseased state, but given its many binding partners, CTGF may have other functions during normal development and disease state.

Importantly, abnormalities in subplate neurons have never been explored in TSC, and their particularly elevated pS6 levels compared to other TSC-deficient neurons merits further study of their contribution to neural development. Excessive postnatal retention of subplate neurons has been shown to result in drug-resistant epilepsy, disturbance in cortical processing, and other long-term neurological

deficits (Cepeda et al., 2007; Bunney and Bunney 2000). The higher number of CTGF-positive subplate neurons and qualitatively thicker layer 6 we observed suggests that there may be an increased number of subplate neurons that persist in the TSC-deficient mutant brains. In addition, subplate neurons express guidance molecules for thalamocortical afferents (“protomap”), intimately tying this layer of cells to the cortical mapping process; therefore upregulation of the cues expressed by the TSC-deficient subplate neurons may cause erroneous pathfinding (O’Leary and Borngasser 2006). It is not yet clear whether TSC patients have mis-wired thalamocortical tracts. Diffusion tensor imaging and tractography studies have recently begun to advance our understanding of the major intrahemispheric tracts by tracking diffusion rates of water through the axons, and showed that geniculocalcarine tract, the corpus callosum, anterior limb of the internal capsule and superior temporal gyrus axon diffusion characteristics were altered in TSC patients (Krishnan et al., 2010; Peters et al., 2012). Although these studies have allowed us to infer the impairments in white matter and disorganization of the axons, the resolution is not enough to discriminate the origin and destination of each of the axonal tracts. Nonetheless, coupled with our understanding of aberrant retinogeniculate projections and disturbed neuronal polarity in TSC mouse models, one can predict that thalamocortical projections may very well be abnormal (Nie et al., 2010; Choi et al., 2008). Although our study only looked at CTGF-positive subtypes, the findings show the need for a more thorough study of subplate neurons in Tsc-mutant brains to delineate the cells’ role in the manifestation of the known neuropathologies associated with subplate neurons.

Beyond the subplate neurons, my work also highlights the need to understand how TSC-deficiency impedes the functions of subtypes of CNS cells. For instance, losing TSC function does not result in the same molecular manifestations across cell types. In fact, loss of TSC in oligodendrocytes does not result in increased levels of CTGF (data not shown), negating the possibility that autocrine-signaling using the same molecule could contribute, and emphasizing that the findings in one cell type cannot be broadly applied without careful investigation.

This is especially true for CNS cells, such as neurons whose morphological characteristics reflect complex intracellular signals that govern cell-specific mechanisms like neuronal polarity and local translation. According to our microarray results, CTGF transcripts in neurons were 2-fold more elevated than those in the soma of TSC-KD neurons, bringing in the possibility of differential translation within a neuron. We have shown previously that the TSC/mTOR pathway can regulate translation of certain proteins in the neurites (Nie et al., 2010). Beyond the disease mechanism, knowing whether CTGF is expressed in the axons and thus concentrated spatially during active OL differentiation and myelination will ascertain its contribution in normal white matter development. We have yet to confirm whether the microarray data translate to elevated protein expression in the axons compared to the soma. However, we know that there is a conserved cytoplasmic polyadenylation element (CPE:UUUUUAU) upstream of a hexanucleotide AAUAAA. These conserved 3-UTRs indicate that CTGF translation may be subject to CPE binding proteins that are expressed in the brain and implicated in localized mRNA translation (Bestman et al., 2008; Si et al., 2003). In

addition to providing additional insights into CTGF modulation alone, this local synthesis hypothesis would provide a new paradigm in axon-glia communication in which necessary proteins are synthesized locally at the site of interaction.

Implications for other diseases/injury

In the CNS, normal neurons, reactive astrocytes, and injured neurons express CTGF. This initially suggested that it may play a role in tissue reorganization following injury, but there has been scant follow up work performed (Kondo et al., 1999; Hertel et al., 2000; Schluesener et al., 2001). In the human brain, CTGF was found to be highly upregulated in Alzheimer's disease brains within neurons and perivascular astrocytes, especially in regions associated with plaques and tangles. (Arendt et al., 2001) This alludes to the role of CTGF in the process of chronic neurodegeneration in AD. CTGF expression in these brains coincided with high levels of TGF- β and VEGF, upstream regulators of CTGF that are closely associated with AD. The importance of CTGF expression in the neurodegenerative environment is unclear. CTGF binds to low-density lipoprotein-receptor-related protein (LRP), and that LRP is critical in numerous neuronal remodeling and synaptic plasticities (Herz et al., 2001; Yosimichi et al., 2001). Given this evidence, it is reasonable to speculate that high CTGF secretion may participate in transport of cholesterol-containing lipoproteins, activation of tyrosine kinases, and mitogen activated protein kinases involved in phosphorylation of tau. Regardless, since CTGF is a secreted protein with multiple binding sites, it can have a broad range of effect at high levels. Our work

showcases one effect on dysmyelination by inhibition of differentiation, but the findings can extend to demyelination models as well.

The importance of myelination for proper function of neurons is irrefutable. There may be other developmental disorders with similar pathogenetic mechanisms and prognosis as TSC that are classified as autism spectrum disorders. TSC is one of the most frequent single genetic cause of autism, given about 50% of TSC patients are diagnosed with ASD (Asano et al., 2001; de Vries et al., 2005). With the multitudes of variations in phenotypic expression across the TSC patients, it is difficult to distinguish which neuropathology is the best indicator of the autistic qualities. However, recent DTI imaging shows that the most remarkable difference in autistic TSC patients brains may be their alterations in white matter microstructural properties, with more prominent changes in TSC patients with ASD than those without (Peters et al., 2012). Therefore, hypomyelination may be an important criterion and indicator for the severity of cognitive deficits in autism and other genetic diseases that involve the mTOR pathway that result in ASD. Such conditions include Neurofibromatosis type 1, Fragile X syndrome, and PTEN hamartoma syndrome, all of which have been associated with ASDs, behavioral dysregulation or intellectual disability. More intriguingly, there are evidences for mTORC1 hyperactivation in focal cortical dysplasias and gangliomas in these other diseases similar to TSC, necessitating a more careful investigation into the changes in white matter as well (Ljungberg et al., 2006; Boer et al., 2010).

Beyond the developmental implications, the current work also provides a possible inhibitory mechanism during remyelination. The regeneration potential of

CNS axons following injury or inflammatory/ autoimmune-mediated damage has been limited at best, and one of the difficult aspects of regeneration has been remyelination. In the case of multiple sclerosis (MS), OPC differentiation is believed to be caused by Jagged1-Notch1 signaling that is triggered by upregulation of TGF- β 1 by microglia and astrocytes during the inflammatory progression of the disease. However, in light of our results, and the fact that TGF- β 1 is a strong inducer of CTGF, the possibility that CTGF can contribute to inhibition of remyelination in MS should be explored (Peress et al., 1996; Grotendorst 1996 and 1997). Another upstream modulator of CTGF is VEGF, and we found evidence in our TSC-deficient neuron cultures that VEGF transcripts are significantly upregulated (Data not shown). Coupled with recent data showing that VEGF levels are upregulated in tubers of TSC patients, VEGF could be an additional upregulator of CTGF in TSC and other injury models (Orlova and Crino 2010).

Whether CTGF influences each of these regeneration and remyelination models has to be tested for compliance with the recapitulation hypothesis of myelin regeneration, which postulates that the mechanisms of remyelination following demyelination are a rerunning of the developmental processes (Franklin and Hinks 1999). This theory holds true in some ways, especially in the model of TSC. We have previously reported with our collaborators that intrinsic mTOR activity is suppressed in adult neurons after development has been completed and cannot be reactivated post-injury. In fact, embryonic neurons display strong mTOR activity that then diminishes in adult neurons, and the remaining mTOR activity in adult neurons is further suppressed by axonal injury through a yet unknown negative

regulator (Park et al., 2008). Consequently, upon conditional deletion of *PTEN* or *Tsc1* in retinal ganglion cells (RGCs), with resulting mTOR activation, crushed axons exhibit robust long distance regeneration and increased cell survival (Park et al., 2008). This regenerative potential is not unique to the optic nerve, and *PTEN* deletion is also able to enhance sprouting and outgrowth of corticospinal neurons following spinal cord injury, ultimately reforming presynaptic structures (Liu et al., 2010). In this way, development, more specifically axon growth, can be recapitulated by reactivation of mTOR. However, whether the regenerated axons find the correct targets and form functional synapses has yet to be investigated since even during development, mTOR activity is definitely not the only element. Adding to the complexity is that other surrounding signals and factors have to become involved for regenerative process to be more successful. It was more recently shown that intraocular inflammation induced oncomodulin and elevation of intracellular cAMP levels, in combination with *PTEN* deletion, are complementary in bolstering the long-distance axon regeneration, showing the necessity for activation of parallel injury response pathways for more extensive regrowth (Kurimoto et al., 2010). In addition to these modulators, we have to take into account the mode injury that can lead to non-developmental signals such as inflammatory signals being elevated, and whether there can be a coordinated method of regeneration to recapitulate the initial development. Regardless, the current knowledge of mTORC1's role in these processes predicts that a more precise and coordinated type of modulation of mTORC1 activity may prove necessary during the regeneration process.

It is not clear from our study whether CTGF has an inhibitory role in the normal developing process or is a disease model-exclusive mechanism applicable to both dysmyelination and demyelination models. Understanding the relationship between the two processes and how the molecules of interest (such as CTGF) apply is imperative to define the treatment and therapeutic time window.

Many TSC mouse models have been created and yielded valuable insights into cell autonomous role of TSC, and our experiments take a novel approach using an existing mouse model to address how TSC-deficiency in one cell type can negatively affect the development of another. Importantly, we show the intimate modulatory relationship between neuronal mTOR and oligodendrocyte mTOR; to our knowledge this is the first evidence of same protein modulation across different cell types. Additionally, the findings of this study bring forth additional perspective on neuropathological manifestations in TSC during development at a molecular level, beyond the past studies that heavily attributed the cognitive deficits on SEGAs, tubers, and more obvious malformations. Hence, our findings warrant careful investigation into finer, more intricate molecular perturbations and neuron-glial interactions in disease mouse models including cell-specific knockout models.

My thesis work provides novel insights into development of hypomyelination in TSC. More importantly, it provides evidence that neuronal signals are critical component of proper development of oligodendrocytes, and outlines the mechanisms that need to be further investigated to uncover the cause of white matter deficits in TSC and beyond.

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Review

TSC1/TSC2 signaling in the CNS

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ABSTRACT

Over the past several years, the study of a hereditary tumor syndrome, tuberous sclerosis complex (TSC), has shed light on the regulation of cellular proliferation and growth. TSC is an autosomal dominant disorder that is due to inactivating mutations in *TSC1* or *TSC2* and characterized by benign tumors (hamartomas) involving multiple organ systems. The TSC1/2 complex has been found to play a crucial role in an evolutionarily-conserved signaling pathway that regulates cell growth: the mTORC1 pathway. This pathway promotes anabolic processes and inhibits catabolic processes in response to extracellular and intracellular factors. Findings in cancer biology have reinforced the critical role for TSC1/2 in cell growth and proliferation. In contrast to cancer cells, in the CNS, the TSC1/2 complex not only regulates cell growth/proliferation, but also orchestrates an intricate and finely tuned system that has distinctive roles under different conditions, depending on cell type, stage of development, and subcellular localization. Overall, TSC1/2 signaling in the CNS, via its multi-faceted roles, contributes to proper neural connectivity. Here, we will review the TSC signaling in the CNS.

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1. Introduction

TSC is a multisystem disorder, in which 90–95% of the affected individuals have CNS symptoms or signs. Neurologically, TSC can manifest with intellectual disability, behavioral abnormalities, autism spectrum disorders (ASD), and seizures [1]. Epilepsy occurs in 80–90% of all patients, often with medically refractory seizures. Close to 45% of patients have mild-to-profound intellectual disabilities and ASD occurs in up to 50% of patients [1,2]. TSC can be diagnosed in the pre- or perinatal period [3], and many neuro-pathological features such as cortical tubers and histological abnormalities are present by the second trimester in utero indicating that neurological manifestations of the disease develop during the embryonic period [4,5]. Clinical signs can be variable with some individuals within a family having minimal symptoms while others carrying the same mutation being severely affected.

The neuropathological findings in the brain usually take the form of (1) subependymal nodules, (2) subependymal giant cell astrocytomas (SEGA) and (3) cortical tubers [6]. Subependymal nodules are lesions found along the wall of the lateral ventricles in the brain. In 5–10% of cases, these benign lesions can grow into SEGAs that block the circulation of cerebrospinal fluid resulting in hydrocephalus. Tubers are made up of a collection of abnormally large neurons and glia and are most commonly found in the

cerebral cortex. It has been proposed that the presence of cortical tubers contribute to the severity of the disorder, but studies have presented conflicting findings on which aspects of the tubers are most critical indicators. More recent studies indicate that the tuber volume is a better reflection of the severity of cognitive impairment than tuber number alone, and also that the location of the tubers (frontal/occipital/temporal/cerebellar) has differential associations with comorbid neuropsychiatric disorders [7–9]. Sophisticated analysis of the neuronal function of TSC1/2 genes in vitro and in animal models has revolutionized our understanding of the disease mechanisms and potential treatment options.

1.1. TSC1 and TSC2 protein complex

TSC1 (on chromosome 9) and *TSC2* (on chromosome 16) are tumor suppressor genes that integrate extrinsic and intrinsic signals of the cellular energy status and growth. Proteins encoded by *TSC1* and *TSC2* genes, also known as hamartin and tuberin, respectively, bind to each other to form a GTPase activating protein (GAP) complex that plays a critical role in the regulation of protein synthesis, controlling cell growth and size [10]. Both proteins are required for the proper function of the complex, and thus a mutation in either gene is sufficient to cause the clinical disease. *TSC1* is required to stabilize *TSC2* and prevent its degradation. On the other hand, the functional GAP domain resides in *TSC2*, making each protein obligatory for each other's functional role. In fact, studies in *Drosophila* have shown that the *Tsc1/Tsc2* double mutants phenocopy either single mutants and that overexpression of both proteins is

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required to render a gain of function phenotype [11,12]. Nonetheless, patients with *TSC2* mutations have a worse overall prognosis than those with *TSC1* mutations [13], and the conditional *Tsc2* knockout mouse model has a more severe phenotype than the conditional *Tsc1* knockout in the same conditional genetic background [14]. These differences could be due to the fact that the two proteins have additional independent functions. Another possibility is that, although both *TSC1* and *TSC2* are subject to ubiquitin-mediated degradation if not bound to each other, some enzymatic activity of *TSC2* remains and is able to carry out some of its function before its degradation. *TSC1*, which has no such catalytic domain, would be ineffective in suppressing mTOR activity on its own [15]. Investigations into the protein interactors of *TSC1/2* have begun in non-neuronal cell lines [16–18], but the question of which proteins interact with the *TSC1/2* complex in CNS cells – at different times during development and at different subcellular locations – has not yet been explored.

The *TSC1/2* complex can be regulated post-translationally by several major signaling pathways in cells: PI3K-Akt, ERK and AMPK (Fig. 1). The best-characterized function of the *TSC1/2* complex is as a downstream target of the phosphatidylinositol 3-kinase (PI3K) pathway that becomes activated upon the binding of growth factors (e.g. IGF or BDNF). Activated PI3K leads to recruitment of PDK1 and the serine/threonine protein kinase Akt, and subsequent phosphorylation/activation of Akt by PDK1. Activated Akt negatively regulates TSC by directly phosphorylating *TSC2* on five consensus sites on human *TSC2* [19–22]. A second kinase that can phosphorylate and inhibit *TSC2* is the extracellular signaling-regulated kinase (ERK) [23]. ERK phosphorylation of *TSC2* appears to be particularly important for EphA-receptor mediated regulation of *TSC2* [24]. Both active Akt and ERK levels are found to be high in *TSC*-related cortical tubers and SEGAs, and the inhibition of *TSC2*

by these kinases has been proposed to represent a post-translational mechanism that may further amplify the loss of the first allele of the *TSC* gene [23,25]. In addition, AMP-activated protein kinase (AMPK) can phosphorylate *TSC2* on a different set of residues than Akt and ERK and potentially increase the ability of *TSC1/2* to inhibit the mTORC1 activity, thereby protecting cells from excessive energy use during low energy states [26,27]. *TSC1* is also negatively regulated by IKK-beta, which physically interacts with and phosphorylates *TSC1* at its Ser487 and Ser511 residues in response to inflammatory pathway activation [28]. Because *TSC1:TSC2* functions as a dimer, regulation of either protein most likely affects its overall activity level. However, relatively little is known about the post-translational modifications affecting the *TSC1* protein and the hierarchy of the regulatory modification on the *TSC1/2* complex, particularly those involving AMPK and IKK. Furthermore, none of these post-translational modifications appear to affect the GAP activity of *TSC2* per se, but they somehow affect the ability of the *TSC1/2* complex to act as a Rheb-GAP in cells. Whether this effect is due to changes in subcellular localization or other cellular mechanisms are not yet clear.

1.2. Downstream of TSC: mTORC1 and 2

When active, *TSC2* inhibits Ras family GTPase Rheb by stimulating the conversion of Rheb-GTP to Rheb-GDP. Downstream targets of Rheb include the serine–threonine kinase mammalian target of rapamycin (mTOR), a central regulator of protein synthesis. mTOR kinase exists in two distinct functional complexes, mTOR Complex 1 and mTOR Complex 2, defined by two groups of binding partners (Fig. 1). mTORC1 is comprised of the core essential components Raptor and LST8, while mTORC2 contains Rictor, LST8, and SIN1. mTORC1 is bound strongly and is quickly inhibited by rapamycin,

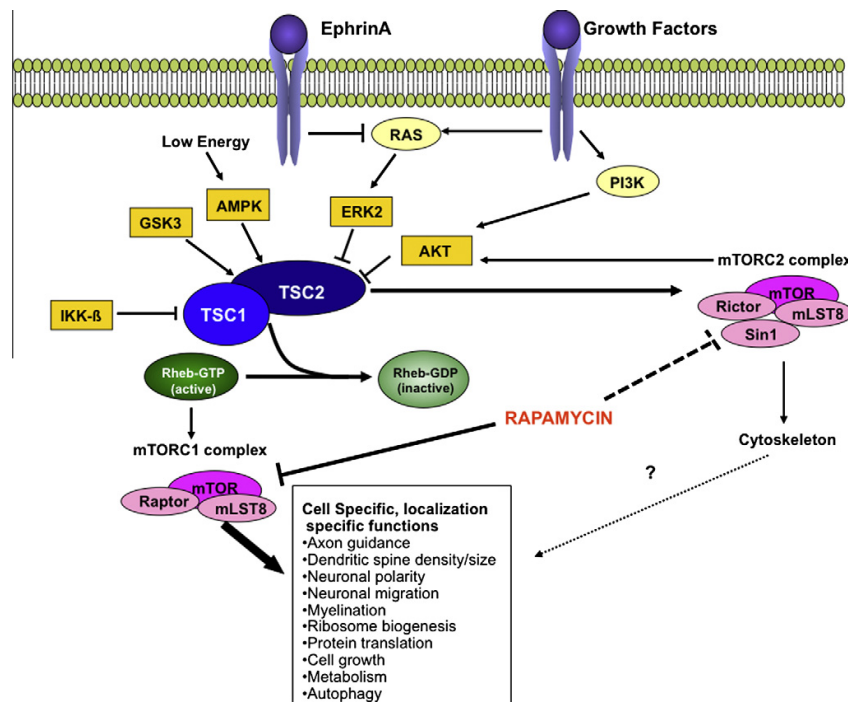


Fig. 1. TSC mediated signaling in the CNS. This cartoon of TSC mediated signaling has been simplified to highlight the demonstrated biologic roles for TSC mediated mTOR signaling in the nervous system.

while mTORC2 inhibition requires prolonged rapamycin treatment, which blocks mTORC2 assembly [29]. However, rapamycin does not fully inhibit mTORC1 function, with some downstream targets being more sensitive than others [30]. mTORC1 phosphorylates and activates ribosomal S6 kinases (S6K1 and S6K2) and inhibits the translational regulator 4E-BP1 – both events that positively regulate translation of 5'capped mRNAs. mTORC1 phosphorylates S6K1 on Thr389, resulting in phosphorylation of its downstream effectors that increase mRNA translation [31]. Activation of S6Ks leads to phosphorylation of ribosomal protein S6, elongation factor 2 kinase (eEF-2K), programmed cell death protein 4 (PDCD4), and eIF4B, all of which result in increased protein synthesis [32–35]. Unphosphorylated 4E-BP1 is bound to eukaryotic initiation factor 4E (eIF4E), inhibiting its association with the eIF-4F cap-binding complex, thereby blocking translation initiation [36]. When phosphorylated by mTORC1, 4E-BP1 dissociates from the eIF4E complex, initiating mRNA translation [37,38]. Thus, without the functional TSC complex, mTORC1 is hyperactive, resulting in constitutively phosphorylated S6 protein, disinhibited protein synthesis, and subsequent cell growth [39,40]. As a central regulator of cell growth, mTORC1 is sensitive to nutrient and redox states of the cells, and more recently has been shown to be specifically responsive to amino acids through a not yet well defined pathway involving the Rag GTPases [41–43]. The presence of amino acids somehow alters the nucleotide-bound state of a heterodimeric Rag complex at lysosomal membranes, and this creates a docking site for mTORC1 [42]. Once at the lysosomal membrane, mTORC1 encounters Rheb, but it is not yet clear how or if Rheb is targeted to the same endomembranes as mTORC1 and Rag proteins. Whether neuronal mTORC1 has specific function at the lysosome has yet to be investigated, and it would be interesting to find out other cellular localization sites of TSC1/2 and mTORC1 and their relevance to the function of this signaling pathway.

Our understanding of mTORC2 is nascent when compared to mTORC1 (especially in the CNS), but it is emerging as a critical component of the PI3K/mTOR pathway. While TSC1/2 negatively regulates mTORC1, it promotes mTORC2 activity in a Rheb-independent manner that might involve the direct binding of the TSC1/2 complex to components of the mTORC2 complex [44–46]. Once active, mTORC2 phosphorylates and activates AKT, leading to phosphorylation of its downstream effectors including TSC2 [47]. There also appears to be crosstalk between mTORC1 and mTORC2, as S6K1 phosphorylates and inhibits Rictor [48]. Interestingly, loss of the TSC1 or TSC2 leads to a unique cellular scenario in which mTORC1 is activated and mTORC2 is attenuated. An important implication of these findings is that the ideal treatment for loss of TSC1/2 may require not only mTORC1 inhibition but also mTORC2 activation. As most of these initial studies were performed in non-neuronal cells, it has yet to be seen whether the pathway is conserved in the CNS and how the intricate balance between the two mTOR complexes affects neuronal function. Studies in the last few years have begun to shed light on the role of these proteins in several aspects of neural development and function, and it is becoming clear that TSC1/2 protein complex is a master regulator of neuronal connectivity.

2. Roles of TSC complex in neuronal development and function

2.1. Axon specification

In the CNS, almost all neurons have a single axon and multiple dendrites. Establishing this unique polarized structure is critical for proper function and directionality of the flow of information within the CNS. Interestingly, TSC pathway components are expressed in neurons in a polarized manner [49–51]. Overexpression of

Tsc1 and Tsc2 suppresses axon formation while loss of Tsc1 or Tsc2 function leads to increased axon number [49]. This critical function of TSC1/2 appears to be related to its ability to regulate levels of a number of proteins such as SAD-A, a kinase required for axon formation in the mouse brain. When TSC is non-functional, SAD-A proteins levels are increased in neurons in an mTOR-dependent manner [49]. Other proteins that are regulated by TSC/mTORC1 include CRMP2, Tau1 and Rap1B, all of which can play a role in neuronal polarity [50,51]. There are likely to be other neurite proteins whose expression is regulated by TSC, and the full repertoire of proteins regulated by TSC/mTORC1 in neurites remains to be identified.

2.2. Axon guidance

One of the most critical steps in neural development is the formation of precise neuronal networks. Importantly, there is growing appreciation of the role of protein translation in axons as a crucial substrate of axonal development. Axons have long been thought to lack active translation as rough ER and polyribosomes are hardly detectable in mammalian axons. However, axons severed from their soma can synthesize proteins readily [52–54] and many functional aspects of the axonal growth cone involve local mRNA translation. It is possible that monoribosome or other functional analogs can be used to synthesize proteins in axons [55]. Using either compartmentalized culture systems or direct laser capture of axons, recent studies have revealed that hundreds of mRNAs are present in axons [56,57]. Blockade of local translation not only affects growth cone collapse and turning in culture systems but also impairs normal axon guidance, circuit development and regeneration [24,58–60]. Indeed, Semaphorin 3A-induced growth cone collapse, which requires local translation of RhoA, is blocked by rapamycin treatment [61]. Many other guidance cues, including netrin-1, slit1, ephrins, regulate axonal protein synthesis through either ERK- and/or mTOR-dependent pathways [24,62,63].

One of the first pieces of evidence for aberrant axon guidance in TSC-deficient neurons came from *Drosophila*. The investigators showed that increases and decreases in TOR signaling via Rheb correlated, respectively with changes in synaptic overgrowth and reduction [64]. Additionally, mutant photoreceptor neurons lacking Tsc1 formed disorganized lamina plexus and aberrant projections into the medulla [64]. More detailed molecular study done using a Tsc2 heterozygous mouse model has shown that haploinsufficiency of Tsc is sufficient to produce aberrant neuronal projections. Axons of retinal ganglion cells find their synaptic targets in the lateral geniculate nucleus of the thalamus by interacting with a group of repulsive axon guidance molecules called ephrins, which bind to cell surface receptors called Eph receptors. Tsc2 heterozygous axons display abnormal growth cone collapse in response to ephrins [24]. Therefore, abnormal collapse of these structures in Tsc haploinsufficient neurons and subsequent incorrect projections into the thalamus show the intimate interweaving of the TSC pathway with Ephrin/Eph pathway, which may play a similar role in other axon projections.

2.3. Synapse formation and function

It has long been recognized that neuronal soma size and dendritic growth positively correlate with innervation and the release of trophic factors. In particular, BDNF is reported to be involved in regulating dendritic complexity and soma size [65]. However, the signaling cascades mediating the effects of such trophic factors are not well understood. Recently, several groups have reported that the PI3K/Akt/mTOR pathway regulates soma size, dendritic arborization and spine morphogenesis [66–68]. Activation of PI3K and Akt both increased cell size and dendritic complexity

while inhibition of endogenous PI3K and Akt decreased cell size and dendritic branching [66,67]. These effects appear to be mediated through mTOR, as treatment with rapamycin or mTOR RNAi decreased dendritic branching [66,67]. Interestingly, Tsc1 or Tsc2 loss increased spine length and head width and decreased the density of dendritic spines in hippocampal slice cultures [68]. Similar decrease in spine density was observed in Tsc1 null neurons in vivo [69].

In addition to structural changes in dendrites, the mTOR pathway is reported to play a role in post-synaptic AMPA receptor expression [70]. In Tsc1 deficient hippocampal neurons, the AMPA/NMDA receptor current ratio was significantly increased relative to that in controls, suggesting an aberrant relative enhancement of synaptic AMPA receptors [68]. In wild-type neurons, the effect of mTOR activation on spine formation appears to be immediate in induction of synapse associated proteins Arc, synapsin I, PSD95, and GluR1 [71]. In this study, the investigators used antidepressant ketamine to activate mTOR, which led to increased spine density and increased EPSCs in response to 5-HT in prefrontal cortical neurons [71]. Although the exact mechanism of how the NMDA receptor antagonist ketamine regulates mTOR activation is unclear, it requires ERK and/or Akt, suggesting that TSC is possibly involved as well. Finally, staining of cortical tubers from TSC patients has indicated a decrease in GluR2 and NR2A staining in giant cells and dysplastic neuron cell bodies [72]. Whether these changes also reflect a reduction in cell surface GluR2 and NR2A expression and the mechanisms leading to these changes are not yet clear. Together, the pre- and post-synaptic roles that the TSC/mTOR pathway plays strongly indicate that abnormalities in this pathway are likely to result in defects in synapse formation, elimination and plasticity, likely correlating with the neurological and developmental symptoms of TSC disease.

2.4. Axon regeneration

Regeneration potential of CNS axons following injury has been limited at best. CNS axon regeneration research has focused on inhibitory factors that have thwarted successful outgrowth of the axons, but recently the TSC/mTOR pathway has emerged as a critical intrinsic modulator of the axon's potential to regenerate after injury. PI3K/AKT pathway is one of the major intracellular responses to neurotrophin regulated axon outgrowth and inhibition of this pathway in neurons reduces the axon growth that occurs in response to growth factor stimulation [73,74]. This intrinsic outgrowth signal may become diminished in adult neurons after development has been completed and cannot be reactivated post-injury. In fact, embryonic neurons display strong mTOR activity that then diminishes in adult neurons, and the remaining mTOR activity in adult neurons is further suppressed by axonal injury through a yet unknown negative regulator [75]. Consequently, upon conditional deletion of *PTEN* or *Tsc1* in retinal ganglion cells (RGCs), with resulting mTOR activation, crushed axons exhibit robust long distance regeneration and increased cell survival [75]. This regenerative potential is not unique to the optic nerve, and *PTEN* deletion is also able to enhance sprouting and outgrowth of corticospinal neurons following spinal cord injury, ultimately reforming presynaptic structures [76]. Since *PTEN* deletion facilitates the regenerated axons' ability to grow slightly more robustly than those lacking Tsc1, it is possible that there are other *PTEN* regulated targets such as GSK-3 β that promote other necessary axon growth functions such as microtubule assembly [77]. It was more recently shown that intraocular inflammation induced oncomodulin and elevation in intracellular cAMP levels, in combination with *PTEN* deletion, are complementary in bolstering the long-distance axon regeneration, showing the necessity for activation of parallel injury response pathways for more extensive regrowth [78].

Nonetheless, the most critical component of the axon regeneration appears to be mTORC1 dependent, probably because of its ability to promote overall protein translation. Whether the regenerated axons find the correct target and form functional synapses has yet to be investigated, but the current knowledge of mTORC1's role in these processes predicts that a more precise and coordinated type of modulation of mTORC1 activity may prove necessary during the regeneration process rather than a complete hyperactivation by *PTEN* suppression.

2.5. Cellular stress

One of the critical homeostatic mechanisms that cells have evolved against intracellular stress is Unfolded Protein Response (UPR). In normal neurons, prolonged chemical induction of ER stress leads to inhibition of the mTOR pathway. Inhibition of PI3K pathway results in increased cleaved caspase-3, reflecting activation of the apoptotic pathway, similar to the recently demonstrated data with Tsc2-deficient cells [79,80]. Tsc loss results in mTOR dependent ER stress response at baseline, and upon treatment with stress-inducing agents such as thapsigargin, the Tsc2-deficient cells exhibit a lowered threshold for induction of UPR-regulated genes and mitochondrial cell death pathways. More importantly, the lack of Tsc activity leads to increased expression of the pro-apoptotic transcription factor CHOP (C/EBP homologous protein), production of reactive oxygen species (ROS), and susceptibility to apoptosis. Immunohistochemical analysis on human TSC brain sections demonstrate similar upregulation in CHOP and heme oxygenase (HO-1), suggesting that heightened ER stress could lead to selective vulnerability of TSC-deficient neurons to extrinsic insults such as seizures, hypoxia, and environmental toxins. Given the reproducibility across cell types on which these studies were performed (neurons, MEFs, kidney cells), it is likely that other CNS cells such as astrocytes and oligodendrocytes would also be susceptible to the damage.

Another cellular response regulated by mTOR is autophagy. Autophagy is an evolutionarily conserved "self-eating" mechanism responsible for the removal of long-lived proteins and damaged organelles by the lysosome. During autophagy, double-membrane autophagosomes sequester intracellular components and then fuse with lysosomes to form autolysosomes in which cargo is degraded. Under growth stimulating conditions, mTOR signaling is activated, which results in inhibition of autophagy. Under starvation conditions, mTOR is inhibited, leading to induction of autophagy. After prolonged starvation, mTOR is reactivated, which reduces autophagy and results in the formation of tubules and vesicles thereby restoring lysosome numbers in the cell [81]. Both ER stress [82] and oxidative stress [83] appear to induce autophagy through the TSC/mTOR pathway. As most of these studies were performed in non-neuronal cells, further studies will be needed to investigate whether TSC1/2 plays similar roles in regulating neuronal autophagy.

3. CNS mouse models of TSC

3.1. Heterozygous models

There are several mouse models of TSC, and although none manifest the full complement of the CNS phenotype in humans – cortical tubers, subependymal nodules or SEGAs – each has provided valuable insight. The first heterozygous mouse models of TSC established that haploinsufficiency of either *Tsc1* or *Tsc2* causes neurocognitive deficits such as impaired hippocampal-dependent learning, social behavior, synaptic plasticity, learning and memory [84,85]. Tsc2 heterozygous mice also exhibit

abnormal mother–pup interaction as measured by ultrasonic vocalizations (USV), establishing these mice as potential models of autism [86]. These neuropsychiatric abnormalities are present without obvious concomitant neuropathological alterations, prompting more rigorous investigation of subtle molecular and circuitry level changes. Heterozygous models display no clear anatomic abnormality under pathologic evaluation, although *Tsc2*^{+/-} neurons do display abnormal axon guidance [24], providing further support for the hypothesis that abnormal neuronal connectivity may underlie the neurological symptoms in TSC disease.

3.2. Neuron-specific models of TSC

Neuron-specific knockout of *Tsc1* in postmitotic neurons has been generated using Cre recombinase under the *Synapsin-1* promoter (*Syn1-Cre*) [87]. *Tsc1*^{fllox/fllox}; *Syn1-Cre* mice are viable perinatally, but develop tremor and hyperactivity beginning the second week of life and die starting approximately 4–6 weeks postnatally. These mice exhibit several neuropathological abnormalities similar to those seen in TSC patients including enlarged dysplastic neurons throughout the cortex, hippocampus, and other subcortical grey matter regions as well as spontaneous seizure episodes. mTORC1 inhibitor treatment has reversed some neuroanatomical abnormalities associated with clinical TSC including reduction in neuron size and improvements in biochemical/signaling profiles, as well as clinical improvements in body weight, clasping behavior, tremor, seizures and kyphosis [39]. Furthermore, when animals are taken off treatment, myelination and other clinical improvements remained intact for at least two more weeks, indicating that regulation of neuronal mTORC1 is critical not only during neurodevelopment but also for long term maintenance of neuronal function.

Deletion of *Tsc2* from radial glial precursors cells using *hGFAP-Cre* transgenic mice results in lamination defects, cortical enlargement, astrogliosis as well as myelination defects [88]. *Tsc2*^{fllox/fllox}; *hGFAP-Cre* mice exhibited severe compromise in survival and profound seizure episodes, suggesting again that cortical tubers are not necessary for the observed phenotypes.

3.3. Astrocyte-specific model of TSC

Another important observation made from *Tsc1*^{+/-} and *Tsc2*^{+/-} mice was the increase in the numbers of astrocytes [89]. As homozygous loss of *Tsc1* or *Tsc2* results in prenatal death, Gutmann and colleagues generated *Tsc1*^{fllox/fllox}; *GFAP-Cre* mice to specifically inactivate *Tsc1* in astrocytes in order to study the impact of the complete loss of Tsc in these cell types [90]. *Tsc1*^{fllox/fllox}; *GFAP-Cre* mice demonstrated up to a six-fold increase in GFAP-immunoreactive cells and subsequent enlargement of some cortical regions such as the hippocampus accompanied by alterations in neuronal organization [90]. Additionally, these mice developed electroencephalographically confirmed seizures by two months of age, but they failed to mimic additional manifestations of TSC such as cortical tubers or cortical lamination defects. The neuropathological phenotypes in these mice were mTORC1-dependent and treatable, as rapamycin treatment prevented development of progressive astrogliosis, abnormal neuronal organization, development of epilepsy and premature death in these mice [91]. Epileptogenesis in these mice was attributed to the increases in extracellular glutamate levels due to decreased astrocytic GLT-1 and GLAST glutamate transporters, and treatment with ceftriaxone to increase the transporter expressions in presymptomatic mice decreased excitotoxic neuronal death and severity of epilepsy [92,93]. Interestingly, when the mice were treated after the onset of seizures, ceftriaxone treatment and subsequent increase in glutamate transporter expression failed to have an effect on seizures, alluding to the importance of early

treatments to prevent permanent neuropathological changes [93]. Furthermore, there may be other astrocytic dysfunction or even embryologic/perinatal alterations arising from TSC-deficiency that may contribute to the seizures given that even the early postnatal treatments did not prevent the seizures completely. Therefore, the exact changes in the neuropathology and the critical time of intervention to effectively prevent seizures require further investigation.

3.4. Role of TSC in oligodendrocytes

Much of the focus in the field has been on astrocytes and neurons, perhaps because of their direct contributions to epilepsy and tuber formation. Nonetheless, the correlation between the severity of cognitive impairments and degree of hypomyelination has brought to surface that the TSC/mTOR pathway is also critical in development and function of oligodendrocytes. Myelination deficits are commonly observed in the TSC brain, both focally within tubers and more diffusely [94–96]. Focal white matter deficits are frequently in the subcortical region underlying tubers, highlighted by ectopic neurons, loss of axons, giant cells, and large astrocytes. More diffusely, this congenital defect persists throughout adulthood, infiltrating numerous major intrahemispheric tracts bilaterally causing approximately 15% reduction in white matter volume [97]. Furthermore, diffusion tensor imaging studies indicate that TSC patients have occult damage in the normal appearing white matter and that this damage may contribute to neurocognitive disability in these patients [96,98–100]. Despite its significance, it has been unclear whether the defect in myelination is cell-autonomous due to loss of TSC function in oligodendrocytes or indirectly due to TSC-deficient neuronal dysfunction.

OLs proliferate, differentiate, and myelinate in independently controlled events that require specific extrinsic cues for each stage of their development. Of these, neuron-synthesized insulin-like growth factor-1 (IGF-1) has been shown to affect all three aspects of OL development [101,102]. Interaction of IGF-1 with IGF-1R on OLs result in rapid transcription and de novo protein synthesis in the OLs through PI3K/Akt, mTOR, and MEK/ERK pathway activation as shown by respective pharmacological inhibition of each pathway component [103]. mTOR activation is required for the terminal differentiation of the oligodendrocyte precursor cells (OPCs) by enabling intrinsic mechanisms to acquire OL-specific gene expression [104]. Interestingly, mTORC1 and mTORC2 appear to have distinct temporal roles in the process; mTORC1 targets P70S6K1 and 4E-BP for phosphorylation at the onset of OPC differentiation while mTORC2 substrate Akt Ser473 phosphorylation was sustained through latter stages of differentiation [104]. Additionally, other recent studies have shown that hyperactivation of the pathway in OL lineage cells either by selective deletion of PTEN (*Olig2-cre*, *Pten*^{fl/fl}) or constitutive overexpression of Akt (*PLP-Akt-DD*) both result in mTOR-dependent hypermyelination [105,106]. PI3K/mTOR pathway appears to regulate myelination by modulating the amount of protein translation; however, this process seems to be limited to the developmental period, as *Olig2-cre*, *Pten*^{fl/fl} mice did not exhibit greater remyelination following lysocleithin induced demyelination [105].

Despite their lack of complete demonstration of the human TSC phenotype, each animal model generated has given valuable insight into the function of the TSC/mTOR pathway in each cell type. Since in the CNS, each neuronal and glial cell type has different functions, it is likely that TSC1/2 play a number of roles in the development and function of each of these cell types. Conditional and inducible knockout of *Tsc1* and *Tsc2* in specific cell types and developmental periods is likely to provide important insights into the CNS biology as well as the pathogenesis of TSC disease.

3.5. TSC-related diseases

Findings from TSC may also have implications for other conditions in which mTORC1 is hyperactive. Such conditions include genetic diseases such as Neurofibromatosis type 1 (NF1), Fragile X Syndrome (FXS), and PTEN hamartoma syndrome, all of which have been associated with ASDs, behavioral dysregulation, or intellectual disability. In addition, there is evidence of mTORC1 activation in focal cortical dysplasias [107] and gangliogliomas [108].

NF1 occurs due to loss-of-function mutations in the NF1 tumor suppressor gene. The NF1-encoded protein, neurofibromin, functions as a Ras-GTPase activating protein (RasGAP). In both NF1-deficient primary cells and human tumors, both ras and mTOR are hyperactivated. In fact, the activation of mTORC1 is dependent on endogenous ras activity in these cells [109]. Furthermore, mTORC1 activity is essential for NF1-associated tumorigenesis [110]. These data suggest that mTOR inhibitors may represent a viable therapy for NF1-related malignancies.

FXS is the most common form of inherited intellectual disability and a leading genetic cause of autism [111]. Accumulating evidence over the last few years indicates that TSC and FMRP pathways interact and share several common signaling components. However, precisely how they interact remains an open question. On the one hand, FMRP can be phosphorylated by S6K1, an enzyme downstream of TSC [112]. On the other hand, mTORC1 is hyperactive in Fmr1 knockout neurons [113], and FMRP-deficient cells display increased activity of PI3K, an enzyme upstream of TSC proteins [114]. These findings have led to the hypothesis that hyperactive PI3K/mTORC1 signaling is pathogenic in FXS [113,115,116]. These important similarities and differences between the two genetic diseases justify further systematic analysis of these conditions using mouse models.

The pathways regulating TSC function have been also implicated in childhood neurological problems, particularly autism. PTEN mutations have been detected in a subset of patients with autism and macrocephaly [117]. Furthermore, when the PTEN gene is deleted in subsets of differentiated neurons in the cerebral cortex and hippocampus, mutant mice show a profound decrease in social interaction and nesting [118]. At a cellular level, PTEN null axonal processes are more exuberant and project to a broader area compared to axons in wild-type mice. Taken together, these results suggest that PTEN inactivation in differentiated neurons is associated with increased axonal growth, ectopic axonal projections, and autistic-like behavior in mice. Importantly, rapamycin treatment blocks the anatomical, cellular, and behavioral abnormalities in these knock-out mice.

4. Future directions

Emerging evidence – from abnormal white matter on neuroimaging of TSC patients to deficits in axonal integrity in animal models – supports the hypothesis that TSC1/2 proteins play crucial roles in neuronal connectivity. In past 20 years since the identification of genetic cause of TSC disease, staggering insights into basic cell biology as well as targeted therapies have been made. Based on clinical trials [119], the U.S. Food and Drug Administration has approved an mTOR inhibitor everolimus in November 2010 for treatment of SEGAs in TSC patients, who are not candidates for surgical resection. At the same time, there is growing evidence that TSC and rheb may have mTORC1-independent functions [120,121]. As we experimentally dissect the TSC/mTOR pathway using more precise genetic tools, the crucial role of this pathway in multiple areas of neural development and function is becoming clear. To accelerate this progress, it will be important to generate animal models that more closely replicate human disease. Major

gaps in our knowledge include: (1) the genetic and non-genetic modifiers of TSC disease that account for the remarkable variability of expression within the human population; (2) cell type and sub-cellular location specific roles of TSC1/2 and their effectors; (3) mTORC1-independent aspects of TSC regulation of neuronal function; (4) the interplay between the different neurological symptoms of TSC disease (epilepsy, autism etc); (5) the relationship between neuronal energetics and the TSC/mTOR pathway. Studies that address such questions will shed light on the interaction between TSC1/2 genes, environment, and neurodevelopment.

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Tsc2-Rheb signaling regulates EphA-mediated axon guidance

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Tuberous sclerosis complex is a disease caused by mutations in the *TSC1* or *TSC2* genes, which encode a protein complex that inhibits mTOR kinase signaling by inactivating the Rheb GTPase. Activation of mTOR promotes the formation of benign tumors in various organs and the mechanisms underlying the neurological symptoms of the disease remain largely unknown. We found that *Tsc2* haploinsufficiency in mice caused aberrant retinogeniculate projections that suggest defects in EphA receptor-dependent axon guidance. We also found that EphA receptor activation by ephrin-A ligands in neurons led to inhibition of extracellular signal-regulated kinase 1/2 (ERK1/2) activity and decreased inhibition of Tsc2 by ERK1/2. Thus, ephrin stimulation inactivates the mTOR pathway by enhancing Tsc2 activity. Furthermore, Tsc2 deficiency and hyperactive Rheb constitutively activated mTOR and inhibited ephrin-induced growth cone collapse. Our results indicate that TSC2-Rheb-mTOR signaling cooperates with the ephrin-Eph receptor system to control axon guidance in the visual system.

Tuberous sclerosis complex (TSC) is an autosomal dominant disease characterized by the presence of benign tumors called hamartomas, which can affect virtually every organ system of the body, including the brain (where hamartomas are known as cortical tubers)¹. Most individuals with TSC also develop epilepsy, and 25–50% are diagnosed with autism spectrum disorders. Although it has been proposed that the cortical tubers cause seizures and cognitive deficits, increasing evidence suggests that there is a poor correlation between cortical tubers and the incidence of epilepsy or autism in individuals with TSC². Furthermore, animal models of TSC have increased susceptibility to seizures in the absence of cortical tubers, supporting the notion that tubers are not responsible for epilepsy. Thus, other mechanisms, such as miswiring of neuronal connections, may contribute to the pathogenesis of epilepsy, autism and intellectual disabilities in individuals with TSC.

TSC is caused by mutations in either of two genes, *TSC1* or *TSC2*, whose protein products form a complex that is important in the phosphatidylinositol 3-kinase (PI3K)-Akt-mTOR pathway. Binding of a growth factor such as insulin to its cell surface receptor leads to activation of PI3K, which in turn activates the Akt kinase. Phosphorylation of TSC2 by Akt releases the inhibitory effect of the TSC1/TSC2 complex on the Ras family GTPase Rheb^{3,4}. Rheb and its downstream effector mTOR are master regulators of cell growth. When Rheb is activated, the protein synthesis machinery is turned on, most likely via mTOR, and cell growth programs are initiated. Therefore, the TSC1/TSC2 complex keeps cell size in check by inhibiting mTOR-mediated mRNA translation. Cells with insufficient TSC1 or

TSC2 function grow beyond their normal size and form hamartomas, but the pathophysiology of the neurological symptoms in individuals with TSC remains poorly understood.

The establishment of neural circuits *in vivo* requires a precise interaction between extending axons and guidance cues in their environment. One of the best-characterized axon pathways in the CNS is the projection of retinal ganglion cells (RGCs) from the eye to their targets in the brain. Many proteins, such as neurotrophins, semaphorins, slits and ephrins, regulate retinal axon pathfinding and topographic mapping in target regions, such as the dorsal lateral geniculate nucleus (dLGN)⁵. Interactions between EphA receptors and ephrin-A ligands expressed in gradients in retinal neurons and across the dLGN are important for initial topographic map formation in the dLGN⁶. Spontaneous retinal activity then contributes to map refinement during postnatal stages^{7–9}.

Binding of ephrin ligands triggers Eph receptor clustering, autophosphorylation and downstream signaling cascades that cause cytoskeletal rearrangements and changes in cell adhesion¹⁰. Through these mechanisms, Eph receptors control axon turning, retraction and branching. Local regulation of protein synthesis and degradation in the axon also contributes to the rapid changes in growth cone dynamics that occur during axonal navigation^{11–15}. Both repulsive and attractive cues can alter local protein translation in an mTOR-dependent manner, suggesting that guidance cues might affect axon growth and navigation at least in part by modulating mTOR activity^{14,16}.

We found a new role for Tsc1/Tsc2 in axon guidance using mouse models of TSC. Components of the Tsc-mTOR pathway were

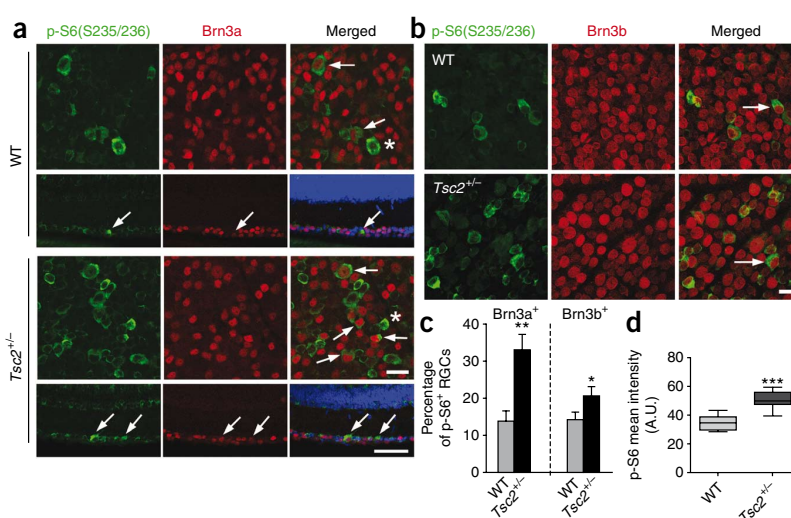
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Figure 1 Increased mTOR activity in *Tsc2*^{+/-} retina *in vivo*. (a) Flat mount (first and third row) or horizontal sections (second and fourth row) of retinas of *Tsc2*^{+/-} mice and wild-type littermates on postnatal day 23 (P23) were stained with antibodies to phospho-S6 (Ser235/236, green) and Brn3a (red). Blue represents nuclear DAPI staining. Arrows denote double-labeled RGCs, and asterisks indicate phospho-S6-positive, Brn3a-negative cells. Scale bars represent 25 μ m in flat mounts and 50 μ m in cross-sections. (b) Flat mount retinas of *Tsc2*^{+/-} mice and wild-type littermates (P23) were stained with antibodies to phospho-S6 (Ser235/236, green) and Brn3b (red). Arrows denote phospho-S6 and Brn3b double-labeled RGCs. Scale bar represents 20 μ m. (c) Quantification of the phospho-S6-positive RGCs as shown in a and b. In both Brn3a- and Brn3b-positive populations, the numbers of phospho-S6-positive RGCs were significantly increased in *Tsc2*^{+/-} mice compared with wild-type littermates (** $P < 0.01$ and * $P < 0.05$ by Mann-Whitney tests).

(d) Quantification of phospho-S6 (Ser235/236) fluorescence intensity as shown in a and b from *Tsc2*^{+/-} versus wild-type (WT) mouse retinas. There was a significant increase in phospho-S6 fluorescence intensity in *Tsc2*^{+/-} RGCs versus wild-type cells. Data are expressed as mean \pm s.e.m. (wild type, $n = 44$ RGCs; *Tsc2*^{+/-}, $n = 114$ RGCs; *** $P < 0.0001$, unpaired t test). A.U. = arbitrary units.



highly expressed in developing RGC axons, and *Tsc2*^{+/-} mice, which have elevated mTOR activity in RGCs, developed aberrant retinogeniculate projections. Consistent with this phenotype, *in vitro* *Tsc2*^{+/-} RGCs were less sensitive to the repulsive effects of ephrin-A. Furthermore, EphA receptor signaling inhibited the mTOR pathway and reduced local protein synthesis in neurons. Our findings reveal a new mode of regulation of the Tsc-mTOR pathway by cell surface receptor tyrosine kinases through the ERK1/2 kinases and shed light on the mechanism by which EphA receptors control mTOR activity and growth cone dynamics.

RESULTS

Increased retinal mTOR activity in *Tsc2*^{+/-} mice

We recently found that components of the Tsc-mTOR pathway are preferentially localized in the axons of embryonic hippocampal neurons and that homozygous *Tsc1* inactivation causes the formation of multiple axons¹⁷. Given that homozygous *Tsc2* knockout results in embryonic lethality¹⁸ and that TSC is an autosomal dominant disease in which most cells in the brains of patients are probably heterozygous for *TSC1* or *TSC2* mutations, we investigated whether *Tsc2* haploinsufficiency might also lead to a multi-axon phenotype. Double-labeling for the axonal marker Tau1 and the somato-dendritic marker MAP2 revealed similar numbers of neurons with multiple axons in cultures of embryonic day 16 (E16) *Tsc2*^{+/-} and wild-type cortical neurons (Supplementary Fig. 1), suggesting that *Tsc2* haploinsufficiency may cause more subtle axonal abnormalities. Immunofluorescence staining of purified RGCs from the early postnatal retina confirmed that, similar to hippocampal neurons¹⁷, wild-type RGCs had higher axonal than dendritic levels of Tsc2 that was phosphorylated at the inhibitory Thr1462 site, total Rheb and active S6K1 (phosphorylated at Thr389) (Supplementary Fig. 2). This indicates that the Tsc2-Rheb-mTOR pathway is preferentially activated in the axonal compartment of different types of neurons, including RGCs. We therefore examined axon guidance and connectivity in *Tsc2*^{+/-} mice, focusing on the developing retinogeniculate projection as a model system.

We first analyzed mTOR activity in the *Tsc2*-deficient retina. To facilitate analysis of RGCs, we stained retinal sections from

postnatal day 23 (P23) mice for β III-tubulin or the RGC markers Brn3a and Brn3b, in combination with phospho-S6 (Ser235/236), a reliable marker for mTOR activity (Fig. 1 and Supplementary Fig. 3). The percentage of Brn3a- or Brn3b-positive RGCs that were also positive for phospho-S6 was significantly higher in *Tsc2*^{+/-} mice than in wild-type littermates ($P < 0.01$ and $P < 0.05$, respectively, by Mann-Whitney test). Quantification of phospho-S6 fluorescence intensity also revealed significantly higher phospho-S6 levels in *Tsc2*^{+/-} than wild-type RGCs ($P < 0.0001$, by unpaired t test; Fig. 1d). Thus, RGCs with heterozygous *Tsc2* inactivation have elevated mTOR signaling *in vivo*.

Abnormal retinogeniculate projections in *Tsc2*^{+/-} mice

To determine whether *Tsc2*^{+/-} mice have axon guidance defects, we labeled RGCs and their axonal projections with the anterograde tracer cholera toxin B (CTB). We injected one eye with CTB-594 (red) and the other with CTB-488 (green) at P14 and visualized the binocular retinogeniculate projections in dLGN sections at P16 (Fig. 2a,b and Supplementary Fig. 4). We noted that *Tsc2*^{+/-} dLGNs occasionally had more than one ipsilateral patch (Fig. 2c), a defect that is similar to that described in ephrin-A2 and ephrin-A5 double-knockout mice⁷. Analysis of ipsilateral and contralateral projections^{19,20} revealed a small, but significant, increase in the percentage of the dLGN occupied by projections from the ipsilateral eye in *Tsc2*^{+/-} mice compared with wild-type littermates ($P < 0.05$, by t test; Fig. 2d). In contrast, similar portions of the *Tsc2*^{+/-} and wild-type dLGN were occupied by contralateral projections or received overlapping inputs (Fig. 2e,f). Using a line-scan technique to calculate the mean pixel intensity along the dorsal-medial to ventral-lateral axes of the dLGN²¹, we found that the ipsilateral projections were shifted toward the ventral-lateral region in *Tsc2*^{+/-} mice (Fig. 2g), similar to what was reported for ephrin-A knockout mice²¹.

The topography of RGC projections to the dLGN is regulated by the opposing gradients of EphA receptors expressed on RGC axons and ephrin-A ligands expressed on target cells^{6,22}. Thus, the retinogeniculate topographic mapping defects in the *Tsc2*^{+/-} mice suggest that RGCs with defective Tsc2 signaling are less sensitive to

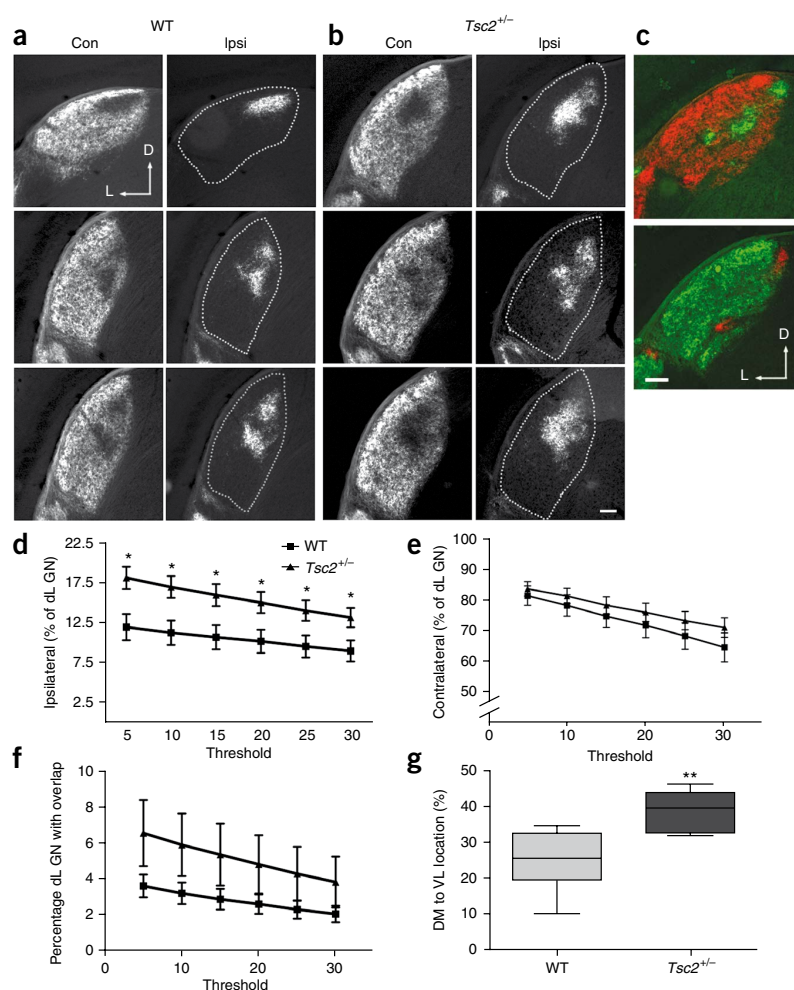


Figure 2 *Tsc2*^{+/-} mice have defects in ipsilateral retinogeniculate projections. (**a, b**) A series of coronal sections from the anterior (top) to the posterior (bottom) showing retinogeniculate projections into dLGN in P16 *Tsc2*^{+/-} (**a**) and *Tsc2*^{+/-} (**b**) mice. Alexa594 (red)- and Alexa488 (green)-conjugated CTB were injected into the left and right eyes, respectively. D, dorsal; L, lateral. Dashed lines represent the borders of dLGN in sections displaying the ipsilateral projections. Scale bar represents 100 μ m. (**c**) In some *Tsc2*^{+/-} mice, ipsilateral projection formed multiple patches rather than a single central patch. Axons from the ipsilateral eye are in green on top and red at the bottom. Scale bar represents 100 μ m. (**d**) Multi-threshold analysis of the percentage of ipsilateral projections in dLGN in wild-type versus *Tsc2*^{+/-} littermates at P16. *Tsc2*^{+/-} mice had larger ipsilateral territories than wild-type littermates, regardless of threshold. Data are expressed as mean \pm s.e.m. ($n = 6$ mice for each genotype, * $P < 0.05$ by t test). (**e**) Quantification of the percentage of contralateral projections in dLGN. No significant difference existed at any threshold between wild-type and *Tsc2*^{+/-} littermates ($P > 0.05$ by t test). (**f**) Quantification of dLGN receiving contra-ipsi overlapping projections. No significant difference existed at any threshold between wild-type and *Tsc2*^{+/-} littermates ($P > 0.05$ by t test). (**g**) The center of the ipsilateral projection in the dLGN was shifted ventrally and laterally in *Tsc2*^{+/-} mice. We used the line-scan technique to calculate the mean pixel intensity along the dorsomedial (DM, 0%) to ventrolateral (VL, 100%) axes in 10 wild-type and 11 *Tsc2*^{+/-} littermates. Error bars represent s.e.m. (** $P = 0.0014$ by Mann-Whitney test).

in the *Tsc2*^{+/-} ventral-temporal projections mimic those observed in ephrin-A knockout mice, suggesting that EphA receptor signaling is attenuated in *Tsc2*-deficient RGCs.

ephrin-A-dependent growth cone repulsion or axon pruning. To distinguish between these two possibilities, we injected eyes with CTB at P4, before the onset of pruning and map refinement⁷. We found that the dLGN area occupied by ipsilateral projections was larger in *Tsc2*^{+/-} mice than in wild-type mice ($P = 0.0017$ by ANOVA, $n = 12$ wild-type and 12 *Tsc2*^{+/-} mice). Although we cannot completely rule out a contribution of pruning or crossing defects, these results indicate that the RGC projections to the dLGN are already aberrantly exuberant before pruning begins.

Because the ventral-lateral shift and the exuberance of the ipsilateral retinogeniculate projections in *Tsc2*^{+/-} mice resemble the defects observed in ephrin-A-deficient mice²¹, we examined the topography of the RGC termination zones in the dLGN. We selectively labeled subsets of RGC axons with focal DiI-crystal placement in the ventral-temporal quarter of the retina. Single termination zones restricted to the dorsal-medial end of the dLGN were observed in wild-type mice, as expected (Fig. 3a). In contrast, the termination zones in *Tsc2*^{+/-} littermates showed a ventral-lateral shift and sometimes had more than one distinct termination zone (Fig. 3b). Quantification of the RGC termination zones along the dorsal-medial to ventral-lateral axis of the dLGN revealed a significantly larger extension of the *Tsc2*^{+/-} RGC projections along the dorsal-medial to ventral-lateral axis compared with controls ($P < 0.01$, by t test; Fig. 3c). Overall, the mapping defects observed

Loss of *Tsc2* inhibits ephrin-induced growth cone collapse

After confirming that *Tsc2* protein expression is reduced in *Tsc2*^{+/-} whole retina lysates and isolated cortical neurons (Supplementary Fig. 4), we used *Tsc2*^{+/-} RGCs to examine the sensitivity of their growth cones to ephrin-induced collapse²³. Ephrin-A1 stimulation caused the collapse of 70–80% of wild-type RGC growth cones, but only 35–40% of *Tsc2*^{+/-} growth cones (Fig. 4a,b). Wild-type and *Tsc2*^{+/-} growth cones collapsed similarly in response to L- α -lysophosphatidic acid (LPA), however, indicating that *Tsc2* deficiency specifically impairs EphA receptor-mediated collapse (Fig. 4b). The decreased sensitivity of *Tsc2*^{+/-} RGCs to ephrin-A is consistent with the aberrant pattern of retinogeniculate projections observed *in vivo*.

To verify that the decreased sensitivity of *Tsc2*-deficient growth cones to ephrin-A1 was not a result of lower EphA expression or signaling, we used a lentiviral shRNA^{17,24} to knockdown *Tsc2* in cultured neurons. *Tsc2* downregulation did not detectably affect EphA4, ephrin-A2 and ephrin-A5 protein levels (Supplementary Fig. 5). Furthermore, cell surface EphA4 level was not significantly affected in *Tsc2* knockdown neurons, as revealed by the cell surface biotinylation assay ($P > 0.05$; Supplementary Fig. 5). Because ephrins induce Eph receptor clustering, which regulates signaling, we also verified that the density of EphA clusters was not altered in *Tsc2* knockdown compared with control lentivirus-infected

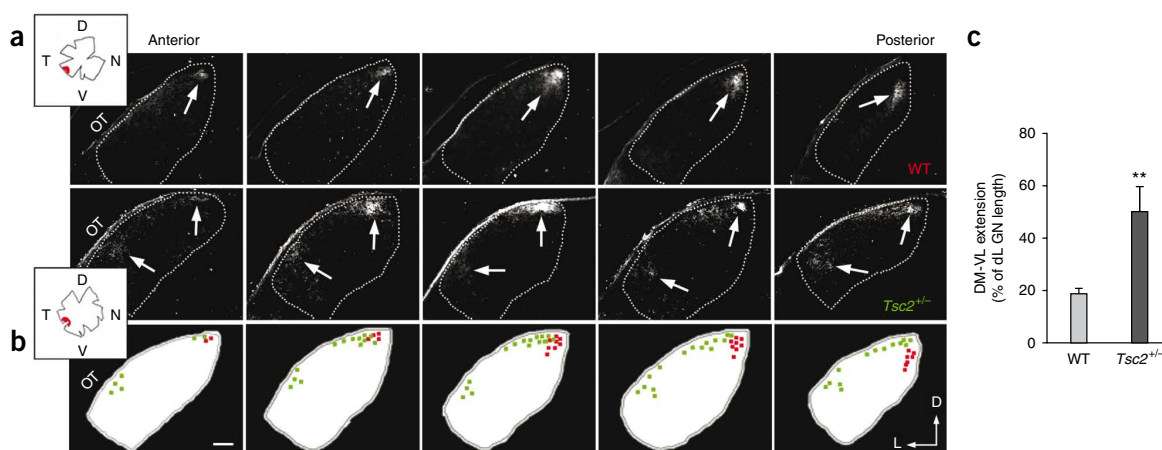


Figure 3 *Tsc2*^{+/-} mice have defects in topographic mapping of contralateral projections in dLGN. (a) Representative series of fluorescent micrographs showing termination territories in contralateral dLGN from Dil-labeled ventro-temporal RGCs of *Tsc2*^{+/+} versus *Tsc2*^{+/-} littermates at P19. Anterior is to the left and posterior to the right in each series of sections. Dashed lines represent the borders of dLGN. Arrows indicate Dil-labeled RGC termination zones. Insets represent tracings of the flat-mounted retina and injection site for each mouse. N, nasal; OT, optic tract; T, temporal; V, ventral. (b) Schematic drawings and colored dots show the position and area of Dil-labeled termination in each corresponding dLGN section from wild-type (red) and *Tsc2*^{+/-} (green) littermates. In *Tsc2*^{+/-} mice, the lower border of the termination extended erroneously into the area that is innervated by the ventral-nasal RGCs in wild-type mice⁸. Scale bar represents 100 μ m. (c) Quantification of the percentages of dorsomedial to ventrolateral extension to length of dLGN along the same axis. Data are represented as mean \pm s.e.m. (19.1 \pm 2.1% in wild type, n = 4 mice; 50.3% \pm 9.7% in *Tsc2*^{+/-} littermates, n = 6 mice; ** P < 0.01 by t test).

neurons (Supplementary Fig. 6). Thus, the effects of *Tsc2* deficiency on the retinogeniculate projection *in vivo* and ephrin-A responsiveness of RGCs *in vitro* were not the results of aberrant ephrin-A/EphA expression, localization or clustering.

Because *Tsc2* is a GTPase-activating protein (GAP) that inhibits mTOR by inactivating the small GTPase Rheb, we also investigated the role of Rheb in growth cone collapse. We used lentiviral vectors to express the constitutively active RhebS16H mutant, which is insensitive to *Tsc2*, and wild-type Rheb, which was also substantially activated

when overexpressed (Fig. 4c). Increased mTOR signaling was verified by measuring the phosphorylation of endogenous S6K1 and S6 in HEK293 cells infected with the Rheb lentiviruses (Supplementary Fig. 7). Ephrin-A1 induced significant collapse in growth cones expressing wild-type, but not constitutively active, RhebS16H (P < 0.05 by t test). However, the collapse was reduced in neurons expressing wild-type Rheb or RhebS16H as compared with control neurons expressing only GFP (Fig. 4d). Therefore, similar to *Tsc2* deficiency, Rheb hyperactivation inhibits ephrin-A-induced growth cone collapse.

Figure 4 *Tsc2* deficiency and hyperactive Rheb inhibit ephrin-A-induced growth cone collapse. Purified RGCs from *Tsc2*^{+/-} and wild-type littermates at P8 were stimulated with LPA (1 μ M) or pre-clustered ephrin-A1-Fc or Fc (control) proteins (5 μ g ml⁻¹). Growth cone collapse was determined by phalloidin staining. (a) Representative micrographs showing staining with rhodamine-phalloidin (red) and antibody to Tuj1 (green) of RGCs with intact versus collapsed growth cone. Scale bar represents 10 μ m. (b) Quantification of the percentage of RGCs with collapsed growth cone (GC). Data represent mean \pm s.e.m. Ephrin-A1-Fc stimulated collapse was markedly reduced in *Tsc2*^{+/-} compared with wild-type RGCs (***) P < 0.001 by t test). There was no significant difference in LPA-treated growth cones from the two genotypes (P = 0.062 by unpaired t test). (c) The lentiviral construct was used to overexpress either wild-type Rheb (wtRHEB) or a constitutively active mutant (caRHEB, S16H), or GFP alone (GFP) as the control in *Tsc2*^{+/-} cells. Western analyses verified higher levels of Rheb, phospho-S6K1 (Thr389) and phospho-S6 (Ser235/6) in wtRHEB- and caRHEB-infected hippocampal neurons than those in GFP-infected or uninfected (NI) neurons, in either 2% (vol/vol) B27 medium or B27-lacking medium for 16 h. Full-length gels are presented in Supplementary Figure 13. (d) Quantification of the percentage of growth cone collapse in purified RGC cultures infected with wtRHEB, caRHEB or GFP lentivirus. Results represent mean \pm s.e.m. from three independent experiments. RGCs with overexpression of wtRHEB or caRHEB had significantly less collapse than GFP-expressing RGCs (by t test, ** P < 0.01 comparing GFP infected cells in Fc versus ephrin-A1 stimulation; * P < 0.05 comparing wtRHEB-infected cells in Fc versus ephrin-A1 stimulation; * P < 0.02 comparing ephrin-A1 stimulations GFP- and wtRHEB-infected, or GFP- and caRHEB-infected, neurons; ns, not significant (P = 0.069) by unpaired t test).

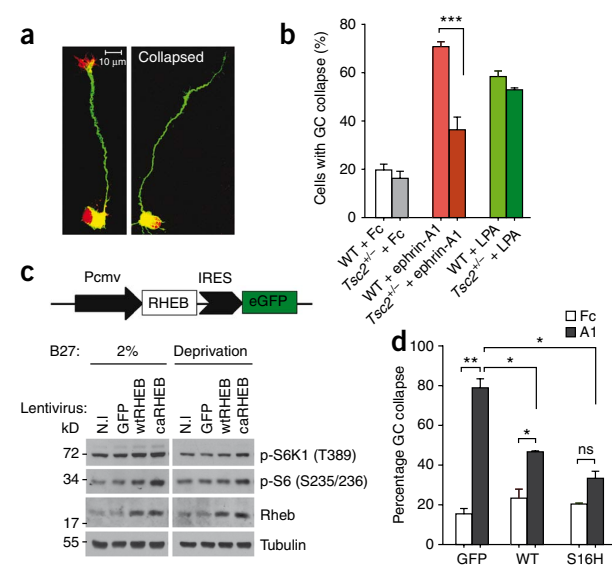
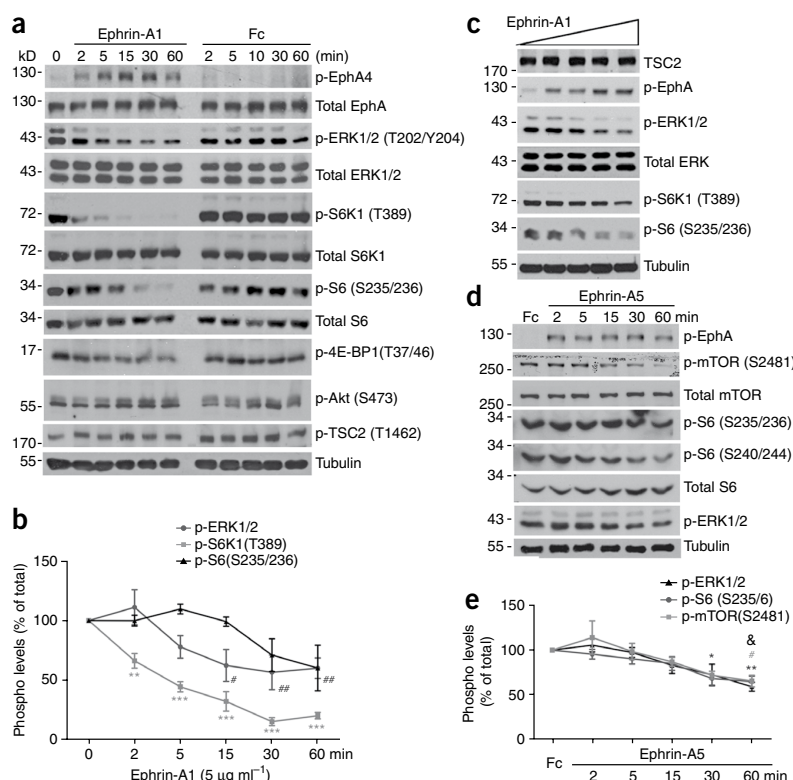


Figure 5 Inactivation of ERK1/2 and mTOR pathway in ephrin stimulated neurons. **(a)** *Tsc2*^{+/+} cortical neurons maintained in culture for 7 d were stimulated with either pre-clustered ephrin-A1-Fc or Fc as a control. Western analyses of neurons stimulated with 5 $\mu\text{g ml}^{-1}$ proteins for the indicated minutes are shown. Total EphA4, ERK1/2, S6K1, S6 and tubulin were used as loading controls. **(b)** Quantitative analysis of phospho-protein levels over the stimulation course as in **a**. Significant reduction in phosphorylation levels was found by *t* test at each time point versus 0 min (** $P < 0.01$ comparing phospho-S6K1 (Thr389) at 2 min and *** $P < 0.001$ at 5, 15, 30 or 60 min; # $P < 0.05$ comparing phospho-ERK1/2 at 15 min and ## $P < 0.01$ at 30 or 60 min). **(c)** Western analyses of cortical neurons stimulated for 30 min with ephrin-A1 at ascending concentrations (0.1, 1, 5, 10 and 20 $\mu\text{g ml}^{-1}$). Total ERK1/2 and tubulin were used as loading controls. **(d)** Western analyses of *Tsc2*^{+/+} cortical neurons grown in culture for 12 d and stimulated with either pre-clustered Fc (as the control) for 60 min or ephrin-A5-Fc for the indicated minutes. Total mTOR, S6 and tubulin were used as loading controls. **(e)** Quantification of the phosphorylation levels of mTOR, S6 and ERK1/2 in Fc versus ephrin-A5-Fc (5 $\mu\text{g ml}^{-1}$) stimulations. A significant reduction in phosphorylation levels was found by *t* test comparing ephrin-A5 at each time point versus Fc stimulation (* $P < 0.05$ comparing phospho-S6 at 30 min and ** $P < 0.01$ at 60 min; # $P < 0.05$ comparing phospho-mTOR at 60 min; & $P < 0.05$ phospho-ERK1/2 at 60 min versus Fc). Full-length gels are presented in **Supplementary Figure 13**.



Ephrin-As depress ERK and mTOR activities in neurons

We next examined whether there is crosstalk between EphA signaling and the mTOR pathway in neurons. Stimulation with ephrin-A1 led to EphA phosphorylation, which was detectable by 2 min and peaked at 30 min post-stimulation (**Fig. 5a**). We also observed an inverse correlation between EphA activation and ERK1/2 phosphorylation/activation, consistent with previous findings in non-neuronal cells²⁵. Ephrin-A1-treated neurons also had rapid dephosphorylation of S6K1 by 2–5 min post-stimulation and a delayed decrease in phospho-S6 after ~15 min of stimulation (**Fig. 5a,b**). 4E-BP1, another effector of mTOR, was also dephosphorylated on ephrin-A1 stimulation. Furthermore, ephrin-A1 inhibited phosphorylation of ERK1/2, S6K1 and S6 in a dose-dependent manner (**Fig. 5c**). Stimulation of cortical neurons with ephrin-A5, the predominant ephrin-A in the dLGN, also decreased ERK1/2, S6K1 and S6 phosphorylation, but with a slower time course (**Fig. 5d,e**). The levels of activated Akt (phosphorylated at Ser473) and TSC2 phosphorylated at Thr1462, a site phosphorylated by Akt, remained constant throughout the period of ephrin stimulation. Thus, activation EphA receptor signaling inhibits both ERK1/2 and the mTOR pathway in neurons, the latter being inhibited independently of the upstream mTOR regulator Akt.

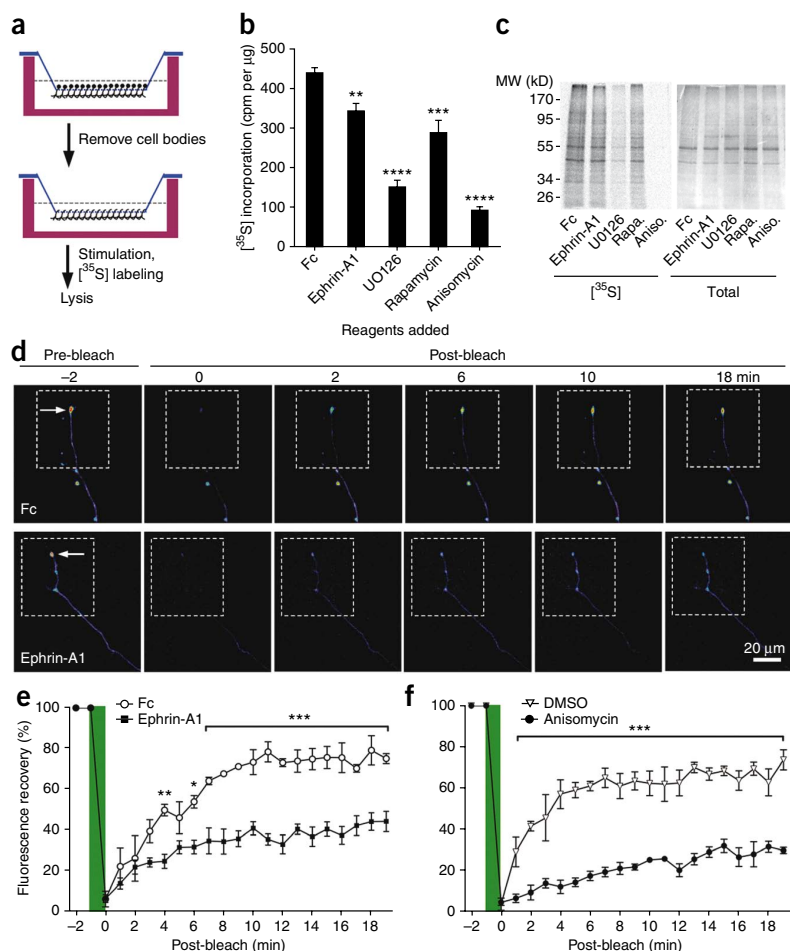
Ephrin-As inhibit protein synthesis in isolated neurites

Inhibition of mTOR activity by ephrin-As would be expected to repress protein synthesis in neurites and growth cones. We used the modified Boyden chamber system to harvest neurite-enriched lysates from neuronal cultures and measure metabolic incorporation of ³⁵S-labeled amino acids into newly synthesized proteins (**Fig. 6a**).

Ephrin-A1 and the mTOR inhibitor rapamycin both caused a similar 25–35% reduction in protein synthesis, whereas translation inhibitors such as UO126 and anisomycin almost completely blocked ³⁵S incorporation (**Fig. 6b,c**). Thus, mTOR inhibition by rapamycin or ephrin-A1 appears to substantially reduce protein synthesis in neurites.

Local translation and degradation of β -actin and proteins that regulate the actin cytoskeleton are important for controlling cytoskeletal dynamics in axons and growth cones^{13–16}. To monitor local translation in ephrin-A1-stimulated neurons, we used the dGFP^{myr}-bACT 3' UTR construct, which encodes a destabilized and myristoylated GFP from a transcript fused to the 3' UTR of β -actin²⁶ (**Supplementary Fig. 8**). This 3' UTR contains a sequence for localization to neuronal processes. In fluorescence recovery after photo-bleaching experiments, we photo-bleached axonal terminal portions, including the growth cones of transfected neurons, and monitored signal recovery over a period of ~20 min. In general, this time scale is not long enough for transport or diffusion of myristoylated dGFP protein from the soma to the growth cone^{27,28}. The dGFP^{myr}-bACT 3' UTR fluorescence signal recovered rapidly after bleaching in control neurons, whereas fluorescence recovery was minimal for the co-transfected control d2-mCherry (data not shown). dGFP fluorescence recovery in axons pre-treated with the translation inhibitor anisomycin (100 μM) was markedly delayed and repressed, as expected (**Fig. 6d–f** and **Supplementary Fig. 8**). Notably, fluorescence recovery in the presence of ephrin-A1-Fc was significantly reduced compared with Fc control ($P < 0.05$ by two-way ANOVA). These findings suggest that ephrin-A1 stimulation inhibits fast and localized translation of β -actin and other mRNAs

Figure 6 Ephrin inhibits axonal protein synthesis and the local translation of a beta-actin reporter in growth cones. **(a)** Schematic representation of the experimental design using [35 S]-labeling and collection of axon-enriched lysates using Boyden transwells (see Online Methods). **(b)** Cell bodies of rat hippocampal neurons grown in Boyden transwells were removed and the neurites in the lower chamber were stimulated immediately with pre-clustered Fc, ephrin-A1–Fc ($5 \mu\text{g ml}^{-1}$), UO126 ($20 \mu\text{M}$), rapamycin (20 nM) or anisomycin ($40 \mu\text{M}$) for 30 min. Quantification of the [35 S] incorporation (cpm μg^{-1}) in trichloroacetic acid (TCA)-precipitated lysate showed that ephrin-A1 inhibited *de novo* protein synthesis as compared with Fc, but to a lower extent than UO126, rapamycin or anisomycin. Results represent mean \pm s.e.m. from three independent experiments (** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ by *t* tests). **(c)** A representative [35 S] autoradiograph showing a reduction in the amount of newly synthesized proteins from axons treated with ephrin-A1, UO126, rapamycin or anisomycin compared with control Fc-treated axons. **(d)** Hippocampal neurons were transfected with dGFP^{myr}-bACT 3' UTR and the dashed lines represent regions of bleaching. Representative images show the fluorescence recovery after photobleaching in terminal axons of transfected neurons over a period of 20 min in the presence of ephrin-A1–Fc or Fc as control. Arrows indicate axonal growth cones. Scale bar represents $20 \mu\text{m}$. **(e)** Quantification of GFP fluorescence intensity pre- and post-bleaching in ephrin-A1–Fc versus Fc treatments. Data at each time point represent average percentage of pre-bleach levels \pm s.e.m. ($n = 3$ for Fc and $n = 6$ for ephrin-A1–Fc). Significant difference in signal recovery was assessed by two-way ANOVA when comparing ephrinA1 versus Fc treatments at each time point post-bleaching (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ at 7–19 min). **(f)** Quantification of GFP signal recovery in anisomycin versus DMSO treatments (*** $P < 0.001$ by two-way ANOVA).



in the growth cone. Nevertheless, we did not detect substantial effects of anisomycin or rapamycin on ephrin-A1-induced growth cone collapse (data not shown), as previously reported²⁹. This suggests that the inhibition of mTOR activity and local translation induced by ephrin stimulation is already sufficient for maximal growth cone collapse.

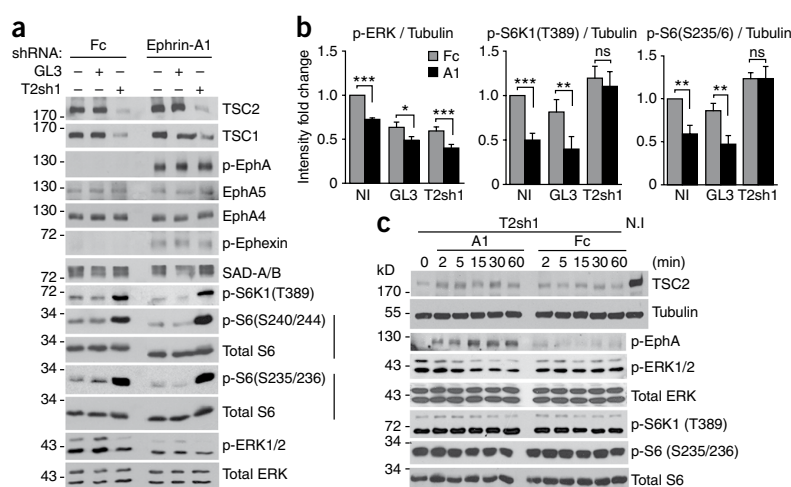
Guidance cues differentially modulate Tsc2 activity

Consistent with its inhibitory effects on ephrin-dependent growth cone collapse, Tsc2 knockdown abolished ephrin-A1-dependent downregulation of S6K1 and S6 phosphorylation in neurons (Fig. 7a,b). The levels of phospho-S6K1 and phospho-S6 remained unchanged over a 60-min time course of ephrin stimulation, excluding the possibility of a delayed dephosphorylation (Fig. 7c). Cre-mediated inactivation of the Tsc1/Tsc2 complex in cortical neurons isolated from *Tsc1^{loxP/loxP}* or *Tsc2^{loxP/loxP}* mice also prevented ephrin-A-dependent S6 dephosphorylation (Supplementary Fig. 9). Furthermore, ephrin-A1 stimulation decreased S6K1 and S6 phosphorylation in wild-type, but not *Tsc2^{-/-}*, mouse embryonic fibroblasts (Supplementary Fig. 10), indicating that EphA signaling can also regulate the mTOR pathway in non-neuronal cells. These results suggest that Tsc2 is both necessary and sufficient for the negative regulation of mTOR activity by ephrin-As,

probably via a mechanism that does not involve Tsc2 phosphorylation by Akt at Thr1462 (see above). Notably, ERK1/2 inactivation by ephrin-A1 still occurred in Tsc2 knockdown neurons, even though baseline ERK1/2 phosphorylation was lower than in control neurons (Fig. 7a,b). Thus, ERK1/2 might be responsible for EphA-dependent Tsc2 activation.

Tsc2 activity can be inhibited not only by Akt-dependent phosphorylation at Thr1462 and Ser939, but also by ERK-dependent phosphorylation at Ser664 (refs. 4,30). Brain-derived neurotrophic factor (BDNF) stimulation markedly increased Akt and ERK1/2 phosphorylation in cultured neurons, whereas ephrin-A1–Fc significantly suppressed ERK1/2, but not Akt, phosphorylation ($P < 0.05$, *t*-test; Fig. 8a). We therefore used phospho-specific antibodies to assess the modulation of Tsc2 by Akt and ERK1/2 in neurons stimulated with BDNF or ephrin-A1. As expected, S6K1 phosphorylation was upregulated by BDNF and downregulated by ephrin-A1 stimulation (Fig. 8a). BDNF also increased Tsc2 phosphorylation of both Thr1462 and Ser664 about twofold compared with control-treated neurons (Fig. 8a,b). In contrast, Tsc2 Ser664 phosphorylation was approximately 35% lower in ephrin-A1-stimulated cells than in control-treated cells. These results suggest that, in cultured neurons, BDNF increases Tsc2 phosphorylation through activation of both Akt and

Figure 7 Tsc2 is required for ephrin-A1–stimulated mTOR inactivation. *Tsc2*^{+/+} cortical neurons were infected with lentivirus expressing shRNA specific for *Tsc2* (T2sh1) or a firefly luciferase (GL3, control). Both uninfected and infected neurons were then stimulated with pre-clustered ephrin-A1–Fc versus Fc for 30 min. **(a)** Representative western blots for indicated proteins. Downregulation of Tsc2 decreased Tsc1 levels and increased SAD-A/B levels, consistent with our previous findings^{17,24}. There were no significant differences in EphA4 and EphA5 expression, ephrin-induced EphA tyrosine phosphorylation or in ephexin phosphorylation between Tsc2 knockdown and control neurons ($P > 0.2$ by *t* test). Tsc2 knockdown abolished the ephrin-A1–dependent downregulation of S6K1 and S6 phosphorylation observed in control neurons. Inactivation of ERK1/2 by ephrin-A1 was still observed in the Tsc2 knockdown neurons, even though the baseline level of phospho-ERK1/2 was lower in these neurons than in the controls. Total ERK1/2, S6 were used as loading controls. Full-length gels are presented in **Supplementary Figure 14**. **(b)** Quantification of the western blot analyses. Relative intensities for each protein were normalized according to the tubulin loading control and expressed as mean ratios of GL3 or T2sh1 to uninfected neurons in each independent experiment. Data represent mean \pm s.e.m. from four independent experiments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and ns = not significant ($P > 0.4$) by *t* test). **(c)** T2sh1-infected neurons were stimulated with pre-clustered ephrin-A1–Fc or control Fc proteins for the indicated time periods. Phospho-S6K1 and phospho-S6 remained unchanged over the time course of stimulation. Tubulin, total ERK, S6K and S6 were used as loading controls.



ERK, which synergistically inhibit Tsc2 GAP function toward Rheb, whereas ephrin-A1 selectively decreases phosphorylation of Tsc2 at the ERK site. Thus, the Tsc-mTOR pathway can be positively or negatively regulated by distinct axon guidance cues.

We then examined whether ephrin-A stimulation can regulate Tsc-mTOR signaling locally in the axon. We detected high phospho-Tsc2

(S664) immunofluorescence in growth cones and filopodia (**Supplementary Fig. 11**). On ephrin-A1 stimulation, the signal diminished rapidly from the growth cone and axon tips. At 5 min post stimulation, when the majority of growth cones (83%) have not yet collapsed, we observed a significant reduction of phospho-Tsc2 (S664) labeling in ephrin-A1–treated growth cones ($P < 0.05$, *t* test;

Figure 8 Regulation of TSC2 (Ser664) phosphorylation by ephrin-A1 is involved in growth cone dynamics. **(a)** *Tsc2*^{+/+} cortical neurons were maintained in culture for 12 d and were stimulated as indicated for 30 min. Phospho-TSC2 (Ser664) or phospho-TSC2 (Thr1462), along with total TSC2, were probed on the same blots and total Akt1 was used as the loading control. Full-length gels are presented in **Supplementary Figure 14**. **(b)** The intensities of phospho-TSC2 (Ser664) and phospho-TSC2 (Thr1462) were normalized to total TSC2. Data are expressed as the percentage of vehicle-treated intensity \pm s.e.m. (* $P < 0.05$ comparing p-TSC2 (Ser664) in ephrin-A1 versus vehicle or BDNF versus vehicle; ** $P < 0.01$ comparing phospho-TSC2 (Thr1462) in BDNF versus vehicle treatment, *t* test). **(c)** Purified RGC cultures from P7 rats were stimulated as indicated and then stained with antibody to phospho-TSC2 (Ser664) and rhodamine-phalloidin. Phospho-TSC2 (Ser664) staining was converted to pseudo-colored spectrum display to distinguish intensity change. Scale bar represents 10 μ m. **(d)** Quantification of pixel intensity of phospho-TSC2 (Ser664) labeling in RGC growth cones. Data represent mean \pm s.e.m. ($n = 4$ –10 growth cones, * $P < 0.05$, ** $P < 0.01$ and ns = not significant ($P = 0.156$) by unpaired *t* test). **(e)** Transfection of neurons with phospho-mimic mutant of TSC2 partially blocked growth cone collapse. Rat hippocampal neurons transfected with wild-type TSC2, mutant TSC2 (S664D/S540D) or empty control plasmids were stimulated with Fc or ephrin-A1–Fc for 30 min. Growth cone collapse was determined by phalloidin staining. Data represent mean percentage collapse \pm s.e.m. from three independent experiments with duplicates in each experiment (* $P < 0.05$, ** $P < 0.01$ and ns = not significant ($P = 0.333$) by unpaired *t* test).

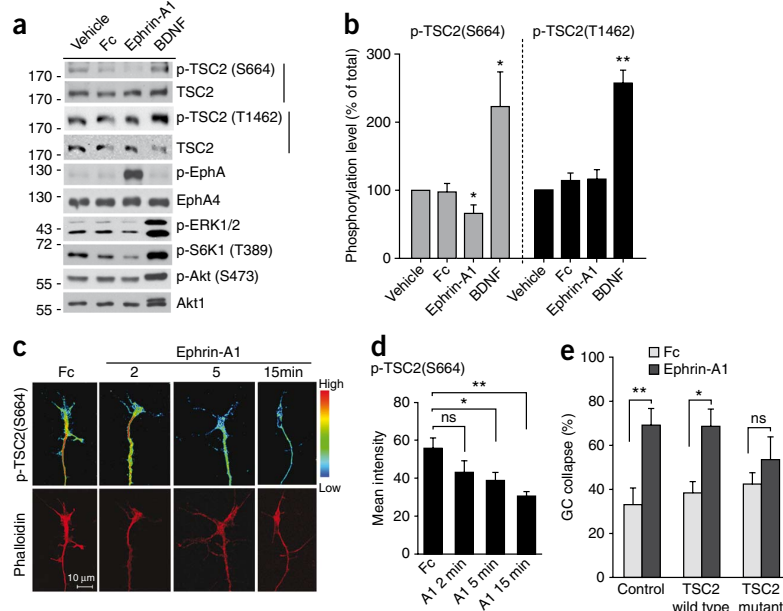


Fig. 8c,d). Phospho-S6K1 immunofluorescence showed a similar reduction after ephrin-A1 treatment (**Supplementary Fig. 11**). Ephrin-A1 stimulation for 15 min led to complete growth cone collapse, coincident with only a faint residual phospho-Tsc2 (S664) and phospho-S6K (T389) signal in the axon tips. Thus, ephrin-A1 induces fast dephosphorylation of Tsc2 on Ser664 and S6K1 on Thr389 in the growth cone, consistent with the timing of inhibition of local translation in growth cones measured with the β -actin reporter.

To confirm that the rapid change in Tsc2 phosphorylation in the growth cone is involved in ephrin-dependent growth cone collapse, we transfected neurons with wild-type Tsc2 or the phospho-mimic S540D/S664D mutant³⁰. We observed a partial blockade of ephrin-A1-induced growth cone collapse by the phospho-mimic Tsc2 mutant, but not by wild-type Tsc2 (**Fig. 8e**). This indicates that ERK regulates Tsc2 activity in the cellular machinery that controls growth cone dynamics in response to ephrin stimulation.

DISCUSSION

We found that the Tsc1/Tsc2 complex was required for ephrin-A-induced growth cone collapse and determined a mechanism by which ephrins can regulate the Tsc-mTOR pathway in axons. We also found that Tsc2-deficient RGCs projected aberrantly to the dLGN, indicating that the Tsc-mTOR pathway is important for Eph-mediated axon guidance *in vivo*. Therefore, Tsc2-mediated regulation of mTOR is crucial for growth cone dynamics and proper axon pathfinding (**Supplementary Fig. 12**).

Eph receptors in neural development, plasticity and repair

The role of Eph receptors and ephrins in the CNS has been extensively studied and a notable feature is the diversity of their functions¹⁰. The Eph receptors represent the largest family of receptor tyrosine kinases in the mammalian genome and regulate various signaling pathways through a number of downstream effectors, including guanine nucleotide exchange factors (Ephexins, Kalirin, Intersectin and Tiam1), GTPase-activating proteins (p120RasGAP, α 2-chimaerin and SPAR), tyrosine kinases (Src, Abl and Fak), phosphatases (LMW-PTP and Ship2) and adaptor proteins (Nck, Grb2, Grb4 and Crk)¹⁰. Here, we discovered a pathway in which Eph receptors regulate mTOR via ERK inhibition, which leads to Tsc2 activation. Eph forward signaling probably simultaneously modulates this and other signaling pathways to modify cytoskeletal dynamics in the growth cone. We found that ephrin-As inhibited mTOR activity in neurons, and it will be interesting to examine whether ephrins of the B class can also regulate mTOR.

Eph/ephrin signaling is important in the formation of not only retinal connections, but also other axonal projections, such as corticospinal and thalamocortical connections and the corpus callosum. Our findings in the retinogeniculate connections may therefore have general relevance for the mapping of axonal connections in the developing CNS. Recently, the Eph system was also shown to be important for radial column formation in the neocortex, implicating these molecules in neuropsychiatric disorders associated with abnormal columnar organization³¹. Whether TSC mouse models have abnormal columnar organization has not yet been investigated. Eph receptors and ephrins also persist in the adult brain, particularly in regions where neuronal circuits continue to be remodeled, such as the hippocampus³². The hippocampal long-term potentiation (LTP) and learning abnormalities reported in Tsc-deficient mouse models could be explained, at least in part, by the involvement of the Tsc-mTOR pathway in Eph signaling³³. Finally, multiple Eph receptors and ephrins are upregulated at sites of CNS injury, where they seem to hinder axon regeneration through their repulsive signaling³⁴. In particular,

EphA4 is emerging as an inhibitor of nerve regeneration, possibly by interacting with both ephrin-B2 in reactive astrocytes and ephrin-B3 in myelin. Recently, mTOR activation by *Pten* or *Tsc1* knockout was shown to promote the regenerative capacity of injured axons³⁵. Our data suggest that one of the possible mechanisms by which mTOR activation could promote axon regeneration is by opposing Eph repulsive signaling. Further experiments involving genetic and chemical manipulations of Eph and mTOR signaling are needed to better delineate their interaction during axon regeneration.

The role of mTOR in axon guidance

We found that, on Eph activation, ERK activity was inhibited. This led to the activation of TSC2, which inhibited Rheb. Consistent with these findings, the loss of Tsc2 and overexpression of constitutively active Rheb decreased the responsiveness of growth cones to repulsive ephrin signals. To date, mTOR is the only known downstream target of Rheb. There are two distinct mTOR complexes: mTORC1, which mainly regulates the translational machinery, and mTORC2, which mainly regulates the actin cytoskeleton. mTORC1 is acutely sensitive to rapamycin, whereas mTORC2 is not, although mTORC2 can be inhibited by long-term rapamycin treatment. Rheb can activate mTORC1 by disrupting the binding of the inhibitory protein FKBP38, but reportedly inhibits mTORC2 (ref. 36). Thus, the similar reduction in growth cone collapse by suppression of Tsc2 or overexpression of Rheb strongly suggests that this phenotype is mediated by mTORC1. Notably, previous studies have shown that growth cone collapse induced by semaphorins or Slit requires rapamycin-sensitive mTOR activity^{16,37}, suggesting that either too much or too little mTORC1 activity may interfere with growth cone dynamics and result in aberrant axon guidance.

The mTOR pathway is a critical regulator of protein synthesis and we found that ephrin stimulation reduced protein synthesis in neurons. However, mTOR might mediate additional functions downstream of Eph receptors, including transcription, ubiquitin-dependent proteolysis, autophagy, membrane trafficking, and microtubule and actin cytoskeleton dynamics. Additional experiments are needed to determine whether the role of mTOR in ephrin-dependent growth cone guidance involves regulation of protein synthesis or additional mechanisms, such as regulation of cytoskeletal dynamics.

Accumulating evidence suggests that the machineries for protein synthesis and degradation are both present and locally active in the growth cone and can be regulated by guidance cues^{11,38}. Axon guidance molecules such as netrin-1 and semaphorins can trigger protein synthesis in retinal growth cones isolated from their cell bodies¹⁶ and inhibition of mTOR has been shown to interfere with proper RGC axon pathfinding. Two recent studies have shown that β -actin mRNA is subject to local translation in the growth cone. In *Xenopus*, the growth cone turning activity of BDNF requires local protein synthesis and is abolished by inhibitors of mRNA translation¹⁵. Local BDNF exposure induces colocalization of β -actin mRNA with the asymmetrically localized ZBP1 protein, resulting in asymmetric distribution of newly translated β -actin and growth cone turning. Similarly, netrin induces transport of Vg1RBP (the *Xenopus* homolog of ZBP1) into filopodia and increases β -actin translation¹³. This requires the 3' UTR of the β -actin mRNA and mTOR activity. Our results suggest further molecular links between axon guidance cues and local mRNA translation in the axon, including the Tsc1/Tsc2 complex and Rheb. Furthermore, we found that ephrin-dependent growth cone collapse involves ERK inactivation and consequent Tsc2 activation, resulting in mTOR suppression. Therefore, the mTOR kinase can be used by diverse extracellular cues to modulate growth cone dynamics.

There are at least two mechanisms by which mTORC1 regulates the translational machinery: phosphorylation of S6K, which increases the translation of mRNA transcripts containing a tract of pyrimidine motif, and phosphorylation of the eIF4E-binding proteins, which relieves the inhibitory effect of 4EBP1 on cap-dependent translation initiation. The roles of S6K and 4EBP1 in axons are poorly characterized, although we recently found that activated S6K1 is preferentially localized in the growing axon when neurites are first developing¹⁷. Identification of the repertoire of mRNAs whose translation is regulated by the Tsc-mTORC1 pathway in axons will provide important insights into the role of translational control in growth cone dynamics.

Pathogenesis of TSC disease

Although substantial progress has been made in identifying the molecular and cellular mechanisms underlying tuber formation in individuals with TSC, an important unresolved issue is what causes seizures and autistic features in these individuals^{2,39–41}. Advances over the past few decades have suggested that dysregulation of axon growth and guidance is important in the pathogenesis of epilepsy, autism and intellectual disabilities. Deficient axonal growth has been associated with cerebral dysgenesis and intellectual disability⁴², whereas excessive growth of neuronal processes has been associated with epilepsy⁴³. The role of neuronal connectivity has also become apparent in autism. Magnetic resonance imaging studies have also demonstrated increased white-matter volume⁴⁴ and aberrant white matter adjacent to brain regions implicated in social cognition in autistic individuals⁴⁵, suggesting that abnormal connections between brain regions involved in social functioning may contribute to impaired social cognition. It will be interesting to see if individuals with TSC and autism can be distinguished from those with TSC, but not autism, on the basis of abnormal connectivity in regions involved in social cognition.

Individuals affected by TSC carry heterozygous mutations in either the *TSC1* or the *TSC2* genes. Loss of heterozygosity (LOH) has been demonstrated in lesions such as cardiac rhabdomyomas, renal angiomyolipomas and subependymal giant cell astrocytomas, but not in cortical tubers⁴⁶. In animal models, acute inactivation of the *Tsc1* gene in hippocampal slice cultures results in increased soma size, decreased spine density and increased spine length in pyramidal neurons⁴⁷. Moreover, *Tsc1*^{+/-} mice have deficits in hippocampal-dependent learning. They also spend less time with unfamiliar mice and are worse at nest building compared with wild-type littermates, two lines of evidence that suggest abnormal social behavior. However, analysis of adult *Tsc1*^{+/-} brains failed to detect any changes in cell soma size, spine density or dendritic arborization⁴⁸. Therefore, the neuropathology associated with the cognitive and behavioral deficits remains unclear. Our data indicate that *Tsc2* haploinsufficiency causes defects in axonal connectivity. It will be interesting to investigate whether the abnormalities that we have detected in the projections of retinal neurons may also be present in other neuronal populations and may contribute to the neurobehavioral phenotype of TSC mouse models and individuals with TSC.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureneuroscience/>.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

D.N. performed most of the experiments. A.D.N. carried out the preliminary biochemistry experiments on the relationship between Eph and Tsc pathways. D.N., I.K., T.H. and M.S. conducted and analyzed the retinal projection experiments. J.M.H. and H.B. performed the quantification on the growth cone collapse assays and immunocytochemical staining. S.D. provided guidance with the initial experiments on the *Tsc2*^{+/-} mice. P.P.P. provided reagents and troubleshooting for experiments on Tsc2 Ser664 phosphorylation. S.C. and E.B.P. contributed unpublished preliminary data on the Eph-Tsc interaction. M.S. supervised the project. D.N., E.B.P. and M.S. wrote and edited the manuscript.

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ONLINE METHODS

Antibodies, constructs and animals. Phospho-TSC2 (S664)³⁰, EphA4, phospho-EphA⁴⁹ and phospho-ephx²³ antibodies have been described previously. Antibodies to phospho-ERK1/2, total ERK1/2, phospho-TSC2 (T1462), Rheb, phospho-S6K1 (T389), total S6K1, phospho-S6 (S235/236), total S6, phospho-Akt (S473), phospho-4E-BP1 (T37/46), phospho-mTOR (S2481) (Cell Signaling), TSC2, EphA4, ephrin-A2, ephrin-A5, EphA5, Brn3b (H-18), (Santa Cruz), phospho-TSC2 (S664) (BioLegend), TSC1 (Zymed), TSC2 (Biosource), Tau1 (Chemicon), MAP2 (sigma), chimaerin1 (Abnova), Brn3a, SAD-A/B (Millipore), recombinant ephrin-A1-Fc, ephrin-A5-Fc (R&D systems) and BDNF (Biosource), and the chemicals PMA, U0126, rapamycin (Sigma) and anisomycin (Calbiochem) were obtained commercially.

The dGFP^{myr}-bACT 3' UTR and d2-mCherry constructs were generous gifts from J. Twiss (University of Delaware) and G Bassell (Emory University), respectively. The lentiviral Tsc2 shRNA construct was custom made by Cellogenetics¹⁷. Tsc2^{+/-} and Tsc2^{0/0} mice were previously described^{18,50}. All procedures were carried out in accordance with the Guide for the Humane Use and Care of Laboratory Animals and our study was approved by the Animal Care and Use Committee of Children's Hospital Boston.

Site-directed mutagenesis. Total RNA was isolated from cultured rat cortical neurons with a Qiagen RNeasy kit according to the manufacturer's instructions. Complementary DNA was then synthesized with Superscript II reverse transcriptase. From the cDNA pool, rat Rheb was PCR amplified with the primer set 5'-AAG GAA AAA AGC GGC CGC ACC ATG CCT CAG TCC AAG TCC CGG-3' (forward) and 5'-CGG GAT CCT CAC ATC ACC GAG CAC GAA GAC TTC CCT TG-3' (reverse). For packaging lentivirus, Rheb PCR product was further cloned into the pHAGE-CMV-IRES lentiviral vector at *NotI/BamHI* sites. Rheb S16H mutant was generated using a QuikChange II XL site-directed mutagenesis kit (Stratagene) with the primers 5'-CCA TCC TGG GCT ATC GGC ATG TGG GAA AGT CCT CAT-3' and 5'-ATG AGG ACT TTC CCA CAT GCC GAT AGC CCA GGA TGG-3'.

Lentivirus infection, neuronal culture and immunocytochemistry. Lentiviruses were pseudo-typed in HEK293T cells that were co-transfected with packaging vectors (rev, tat, gag/pol, vsv-g) and each lentiviral construct. Transfection solutions were replaced 6 h later with DMEM containing 1% FBS. Viral supernatants were collected twice at 48 and 60 h after transfection, filtered and frozen.

RGCs were prepared from P7 rat or P8 mouse pups according to the immunopanning method as described previously²³. Cortical and hippocampal neurons were isolated from E18 rat or E16 mouse embryos. Briefly, cortex and hippocampus were dissected out and dissociated in papain for 5 min in HEPES-buffered Hank's solution. Cells were grown on poly-D-lysine and mouse laminin-coated glass coverslips or only poly-D-lysine-treated plates/dishes in Neurobasal medium with B27 (Invitrogen). The day after plating, neurons were infected with lentivirus in the presence of 0.6 $\mu\text{g ml}^{-1}$ polybrene for 6 h. To overexpress Rheb, we infected the neurons twice at the second and third day after plating.

For immunocytochemistry, neurons were fixed with 4% paraformaldehyde (vol/vol)/4% (wt/vol) sucrose in phosphate-buffered saline (PBS) for 10 min. Fixed cells were permeabilized in 0.1% (vol/vol) Triton X-100 for 15 min and blocked in 4% (vol/vol) normal goat serum and 2% (wt/vol) BSA in PBS. Typically, neurons were incubated with the primary antibody at 4 °C overnight and fluorescent secondary antibody for 2 h at room temperature (22–25 °C). Images were captured using a Zeiss LSM5 confocal microscope system.

Nucleofection. E18 rat hippocampal and cortical neurons were transfected immediately after dissociation using Amaxa Nucleofector (Amaxa Biosystems) system and plated at 50,000 neurons per 13-mm² coverslip. Neurons were used for growth cone collapse assay or live imaging 2 d after plating and transfection.

Ephrin-A1 stimulation and growth cone collapse assay. Ephrin-A1 stimulation and quantification of growth cone collapse was performed as described previously²³. Briefly, to pre-aggregate proteins, recombinant ephrin-A1-Fc, ephrin-A5-Fc (R&D system) or human IgG Fc (Jackson ImmunoResearch Labs) was incubated with goat antibody to human Fc (Jackson ImmunoResearch Labs) at ratio of 1:4.5. The volume was adjusted with HBSS to 5 \times . After shaking for 45 min at room temperature, proteins were diluted in conditioned culture media

and added to the neuronal cultures. Generally, cells were stimulated for 30 min before lysis or fixation unless otherwise specified. LPA-stimulated collapse was performed at 1 μM for 10 min before fixation. To visualize growth cone collapse, we stained fixed neurons with rhodamine-phalloidin (Cytoskeleton). Growth cones that had not collapsed were identified by the presence of lamellipodia and two or more filopodia. Collapsed growth cones were distinguishable from their noncollapsed counterparts by the presence of a collapsed bulb and having fewer than two filopodia at the leading edge. Counting of growth cone collapse was performed blind to genotype or treatment.

EphA receptor clustering study. Hippocampal neurons infected with GL3 against luciferase or TSC2 shRNA (T2sh1) lentivirus were treated with pre-aggregated ephrin-A1-Fc versus Fc (1–2 $\mu\text{g ml}^{-1}$) for 5 min at 37 °C. Neurons were fixed, washed, permeabilized (0.1% Triton X-100) and stained with cy3-conjugated antibodies to human IgG Fc and Tau-1. EphA clusters were imaged with Leica LSM5 confocal system. EphA clusters/punta were counted blind to treatments.

CTB injection and Dil labeling. CTB labeling and analyses were performed as previously described²⁰. Briefly, mouse pups at P14 were injected binocularly with fluorescence-labeled CTB subunit (Alexa 488 or Alexa 594, Molecular Probes) and allowed to recover for 48 h. Dissected brains were fixed in 4% paraformaldehyde for 48 h at 4 °C. Coronal sections (100 μm) were imaged with a 10 \times objective. All of the image acquisition and analyses were performed blind to genotype. The same gains and image exposure times were used for each label in all specimens to limit variability caused by imaging procedures. Raw images of the dLGN were imported to Adobe Photoshop. The size of the ipsilateral, the contralateral and the degree of overlap were quantified with the multi-threshold protocol, which is designed to compare overlap across a range of signal-noise values in control versus mutant mice^{19,20}.

To compare the distribution of the ipsilateral projection pattern, we employed a line-scan technique to calculate the mean pixel intensity of projection terminals in wild-type and Tsc2^{+/-} mice along the long (dorsomedial to ventrolateral) axis. The location of the center of the ipsilateral projection was calculated with the dorsomedial border of the LGN set as 0% and the ventrolateral border as 100%.

To focally label RGCs in the ventro-temporal quarter of retina, we embedded a ~1-mm³ Dil crystal (Molecular Probe) below the cornea-sclera border along the ventrotemporal→dorsonasal orientation into the right eyeball. Mice were killed 5 d later and intercardially perfused with 4% paraformaldehyde in PBS. The eyeballs were extracted and post-fixed in 4% paraformaldehyde for 1 h and the brains were post-fixed overnight. Retinal whole-mounts were dissected out to determine Dil labeling position and area. Brains corresponding to retinas that had comparable Dil labeling were further sectioned with a Leica cryostat and the contralaterally labeled dLGN serial sections were imaged with a Nikon fluorescence microscope with a 10 \times objective and mounted Hamamatsu ORCA-ER camera. Further image processing was performed using Adobe Photoshop and the dorsomedial-ventrolateral extension of dLGN labeling was measured using ImageJ (US National Institutes of Health).

[³⁵S] metabolic labeling of nascent proteins. *De novo* protein synthesis was quantified by measuring the incorporation of ³⁵S-Met/Cys (Perkin Elmer) in TCA-precipitated proteins. Cortical neurons (12 d *in vitro*) in Boyden transwells were washed with and switched to Met/Cys/serum-free DMEM (Invitrogen) for 30 min at 37 °C to deplete intracellular methionine and cysteine. Prior to stimulation and/or ³⁵S labeling, cell bodies were removed in the top chamber and further washed with equilibrated Met/Cys/serum-free DMEM. The bottom chamber was incubated with pre-clustered Fc or ephrin-A1-Fc (5 $\mu\text{g ml}^{-1}$) in the presence of ³⁵S-Met/Cys (40 $\mu\text{Ci ml}^{-1}$) for 30 min or with U0126 (20 μM), rapamycin (20 nM) or anisomycin (40 μM) for 10 min before adding ³⁵S-Met/Cys for another 20 min. Axon-enriched lysates were harvested in 100 μl of RIPA buffer (with protease inhibitors and phosphatase inhibitors). We precipitated 30 μl of each lysate with 1 ml of 10% (vol/vol) TCA and protein pellets were further dissolved in 150 μl NaOH (1 N, 37 °C) and pH-adjusted by adding 50 μl of 0.33 N HCl. Three 50- μl aliquots were each added to 10 ml of Ultima Gold XR (Perkin Elmer) scintillation fluid and read by a liquid scintillation counter. We obtained cpm per μg of protein by dividing the average cpm of triplicates by protein amount (μg) measured with Bradford kit (Bio-Rad). Lysates were also added to 3 \times loading buffer and boiled for 5 min. The gel samples were then resolved on a 10%

SDS-PAGE gel, fixed in 3% glycerol/10% glacial acetic acid/20% methanol (all (vol/vol)) for 1 h and stained with EZblue solution (Sigma) to confirm the equal loading. Gels were then dried completely (80 °C for 2.5 h) and exposed to a storage phosphor screen for 3 d. [³⁵S] autoradiographs were scanned to ImageQuant 5.0 (GE Healthcare) with a PhosphorImager (Molecular Dynamics).

Live-cell imaging and fluorescence recovery after photobleaching (FRAP). E18 hippocampal neurons were transfected with a destabilized GFP containing a myristoylation sequence and further fused to 3' UTR of β -actin (dGFP^{myr}-bACT 3' UTR) and d2-mCherry constructs (at ratio 4.5:3) using Amaxa nucleofection. The beta-actin 3' UTR drives axonal expression of dGFP²⁶ and the myristoylation sequence restrict side diffusion of the GFP on translation. For live imaging, transfected neurons on 35-mm bottom glass dishes (MatTek) were washed and switched to pre-equilibrated Hibernate E Low Fluorescence medium (Brain Bits LLC). FRAP was performed using a Zeiss LSM5 upright confocal microscope fitted with a heating stage and 63 \times water-immersion objective (numeric aperture = 0.9). Pinhole was set to \sim 7.05 for 488 line and \sim 6.33 for 568 line (corresponding to \sim 5- μ m optic slice) to ensure signal collection from entire thickness of axons.

A rainbow spectrum display was employed to adjust the gain and off-set to ensure that the highest pixel intensity is just saturating. Chosen axons were first pre-bleached by scanning (zoom = 0.9) at 31 s per frame with 29-s intervals for 10 frames (\sim 10 min) using 15% laser power (488 line). The region of interest containing axon terminal/growth cone was bleached using 100% laser power for 120 iterations (<2 min), followed by regular imaging using 15% laser power at speed of 7.86 s per scan with a 52.3-s interval over a 20-min course. For guidance cue stimulation, pre-aggregated Fc or ephrin-A1-Fc was added at 5 μ g ml⁻¹ final concentration immediately before imaging and FRAP. For the study of translation inhibition, neurons were pretreated with 100 μ M of anisomycin or DMSO for 20 to 30 min before FRAP study. Fluorescence intensity was measured using ImageJ and statistic analyses were performed in Prism 5 using two-way ANOVA to compare significant signal recovery over the course of treatment and among different treatments.

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